Optimization of Novel SNP DNA Markers for Early Selection of Blight Resistant Hybrid Chestnut in Indiana and Virginia Backcrosses and Grafting to Facilitate Breeding and Germplasm Conservation

HTIRC / IN-TACF FINAL REPORT – J.R. McKenna & N. LaBonte

ABSTRACT

We screened a variety of hybrid chestnut trees from the IN-TACF State Chapter and from the national TACF Meadowview, VA breeding orchards using a set of newly developed SNP markers from predicted Chinese chestnut (*Castanea mollissima*) blight resistance candidate genes that exhibit different alleles in American chestnut (*C. dentata*). Both resistant and susceptible trees were included in each test. Resistance scores were based on the canker rating of 4 to 16-year-old trees in the field, previously inoculated with the *Cryphonectria parasitica* (*Cp*) isolates SG and Ep 155 with a few exceptions. Susceptible trees were collected within and among families of similar aged progeny for each hybrid type, typically from root sprouts.

In our first test, we extracted DNA from dormant twigs of American, Chinese, F1, BC1F1, BC3F2 and BC3F3 chestnuts to validate further the original set of 10 SNP's as well as a dozen more candidate SNPs under development. Next, we compared a sample of both resistant and susceptible and BC3F3 families and individuals from Indiana and Virginia respectively. In order to identify additional loci that may contribute to blight resistance in BC3 generation hybrid chestnuts, we sequenced two pools (10 individual trees per pool) of resistant and two pools of susceptible BC3F3s, with one individual BC3F1, a pool of F1 interspecific hybrids, and a pool of Chinese chestnuts to serve as controls with known allele frequencies. Pooled sequencing allows for a bulked-segregant-like analysis of breeding materials. In all tests, *Cm* alleles have proven to be strongly associated with blight resistance and the corresponding *Cd* alleles have been strongly associated with susceptibility across these loci. These SNP markers should help selecting durable blight resistance since they are based on trees with inoculated stems and natural disease pressure that have survived for at least 16 years without dying back to the ground.

We included a grafting component to this study to aid both the breeding and the conservation of American and hybrid chestnut germplasm. We grafted the most resistant BC3F3 individuals from the eight most resistant families of a progeny test, growing under a natural blight epidemic, onto both BC3F3 and BC1F1 rootstock with 60% success on each rootstock type. Scion wood from surviving Alabama and Tennessee American trees was grafted onto 1-0 American seedlings (whip grafts) in the greenhouse and on 4-year-old American seedlings in the field (bark grafts). Bark grafting on the established rootstocks produced initial graft takes over 70% while Americans on 1-0 seedlings grafted just below 20% in the greenhouse. We also grafted BC3 selections onto both BC3 and BC1 rootstocks in the greenhouse with 60% success.

Taken together, our new SNP markers along with those previously developed by TACF and others, can provide good marker assisted selection tools to identify better parents that can be grafted and developed into new productive breeding and conservation orchards to provide seed for future hybrid American chestnut breeding and ultimately restoration.

Nick LaBonte[1], Aziz Ebrahimi[2], Jared Westbrook[3], James McKenna[4], Shaneka Lawson[4], Dana Nelson[5], and Bob Wagner[2]

[1]Eureka College, Department of Biology, 300 E College Ave, Eureka, IL, 61530; [2]Purdue University, Department of Forestry & Natural Resources, 715 W State, West Lafayette, IN, 47907; [3]TACF, 50 N Merrimon Ave, Suite 115, Asheville, NC, 20804; [4]USDA Forest Service, Hardwood Tree Improvement & Regeneration Center (HTIRC), 715 W State, West Lafayette, IN, 47907; [5]USDA Forest Service, Forest Health Center, 730 Rose, Lexington, KY, 40546

INTRODUCTION

Chestnut blight is the most destructive disease of chestnut (*Castanea* spp.) worldwide and has functionally eliminated American chestnut from its native range (Anagnostakis 1987). The important cultural and economic role of American chestnut as a consistent producer of highly palatable mast for wildlife, decay-resistant timber, and now more recently carbon sequestration (Jacobs 2007), has led to considerable interest in developing blight-resistant chestnuts to restore it in the eastern United States. A backcross breeding strategy (Burnham et al. 1986), led by the American Chestnut Foundation, has been developed to incorporate blight resistance genes from Chinese chestnut into American chestnut but after several decades of breeding, not enough resistance has been captured.

Resistant reactions seem to depend on the rapid development of a lignified callus zone around the infection site (Hebard et al. 1984). American chestnut displays lethal susceptibility to the disease; stems are killed back to the root collar and may re-sprout but there is no evidence of resistance to blight within the species. However Chinese and other Asian chestnut species (*Castanea mollissima*), have coevolved with this blight pathogen and they display quantitative variation in resistance: some trees are nearly immune, while others suffer the loss of major scaffold limbs. Interspecific hybrids are variable in resistance. Quantitative trait loci (QTL) mapping conducted with hybrids identified three major blight resistance loci that together explained about 75% of the variation in resistance among hybrids (Kubisiak et al. 1997, 2013). Several transcripts are up-regulated in blight-infected stems of Chinese chestnut but not in American chestnut, and vice versa (Barakat et al. 2009, 2012), indicating differences in the molecular pathways of disease response between the species. The specific genes and molecular mechanisms that lead to resistance and susceptibility, however, remain unknown.

Understanding which genes are required for hybrids to display similar resistance to Chinese chestnut is important from a practical perspective, because it would allow more informed selection of backcrossed hybrid parents. From a basic scientific perspective, unraveling the molecular basis of blight resistance and susceptibility in chestnut would be a major breakthrough because it could serve as a model for two poorly understood plant pathological phenomena: 1) the molecular nature of resistance to necrotic canker-forming pathogens in woody plants and 2) the extreme disease susceptibility of native hosts to exotic forest pathogens.

A Chinese chestnut reference genome is nearly complete, and the physical and linkage maps have been integrated (Fang et al. 2013, Staton et al. 2015). This has enabled the use of whole-genome resequencing and association genetics to identify blight resistance candidate genes (LaBonte 2017). Nick LaBonte's dissertation work identified several new blight resistance loci, in addition to likely candidate genes for three major QTL already identified (Kubisiak et al. 2013). These candidate genes encompass a variety of predicted molecular functions, including preformed defenses, hormone signaling and metabolism, and some membrane-bound, disease-resistance proteins. The latter category included a cluster of predicted NBS-LRR-type genes,

and a cluster of receptor-like kinases (RLKs), similar to a highly conserved cluster of rust resistance genes in the Poaceae. Rusts are biotroph pathogens, and NBS-LRR genes have not generally been associated with resistance to necrotroph pathogens. Notably, the predicted NBS-LRR and RLK genes showed elevated expression in American chestnut affected by chestnut blight, but not in Chinese chestnut (Barakat et al. 2012). Furthermore, the predicted NBS-LRR genes were also differentially expressed in response to the hemibiotroph *Phytophthora cinnamomi* in European (*Castanea sativa*) and Japanese chestnut (*Castanea crenata*) (Serrazina et al. 2015). Because effective disease responses to biotrophs, which parasitize living cells, often involve programmed cell death (the hypersensitive response, or HR), necrotroph pathogens, which feed on dead cells, may exploit biotroph resistance pathways to cause disease. We hypothesize that the extreme susceptibility of American chestnut to chestnut blight may be due, in part, to a failure to regulate programmed cell death in HR pathways that have evolved in response to biotroph pathogens and are exploited by chestnut blight. Based on association results from Nick LaBonte's 2017 PhD dissertation, and transcriptional evidence from previous studies (Barakat et al. 2012, Serrazina et al. 2015), it is possible that genes downstream of the membrane-bound receptors (e.g., NBS-LRR and LRK) candidate genes "rescue" Chinese chestnut by modulating the resistance pathways that cause susceptibility in American chestnut.

Marker assisted selection is a modern breeding tool whereby plant breeders can probe their parental selections for genes, either simple sequence repeats (SSR's) or single nucleotide polymorphisms (SNPS). Both SSR's and SNP's are two of the most widely used DNA markers in cereal crops (Collard and Mackill, 2008). However, pedigreed and well characterized phenotypes of the trait under selection must be compared and contrasted to discern useful markers. Short term resistance screening has resulted in many selections that ultimately have succumbed to blight, indicating insufficient resistance. With TACF Meadowview, VA and the IN-TACF screening blocks now maturing, we finally have well characterized blight resistant hybrids to develop and test such markers.

METHODS

SNP marker development to assist blight resistance breeding in backcrossed chestnut populations

Summary

We developed 10 SNP markers from predicted genes of Chinese chestnut that exhibited distinct alleles fixed in the American and Chinese chestnut species and one allele from each species in 'Clapper,' the BC1 tree that is the resistance donor for most of the Indiana American

Chestnut Foundation's blight-resistance breeding program. A panel of individual Chinese and American chestnut genomes (sequenced with funds from a previous TACF grant) was used to identify SNP loci throughout the genome that have one allele fixed in all Chinese chestnuts surveyed and another allele fixed in American chestnuts. When this type of SNP locus occurs in or near a predicted gene sequence, it is of particular interest because it may represent a nucleotide change that contributes to differences in the species' phenotypes. Of the 10 loci, genotypes were recovered for nine using low-cost next-generation DNA sequencing (WideSeq) and seven were polymorphic and simple to score. The panel of samples screened included several resistant, moderately resistant, and susceptible BC3F1 chestnuts, American and Chinese chestnut controls, and an F1 control. In general, resistant BC3F1 trees had the highest proportion of loci with hybrid genotypes, and the SNP loci tested show potential for use in marker-assisted selection. This potential should be further investigated by screening a larger population of BC3F1 and BC3F2 trees. Additional SNP markers should also be developed 1) to target genes potentially underlying the cbr3 blight resistance QTL on linkage group G; 2) to serve as non-blight-associated controls; and 3) to screen F1 and BC1 progeny for blight loci with no Chinese chestnut allele present in 'Clapper.'

SNP development

Polymorphisms were selected from introns and exons of genes predicted by AUGUSTUS gene prediction software (Stanke et al. 2005) in the draft genome of Chinese chestnut (*Cm*). The draft genome sequence (beta version of pseudochromosome assembly reference sequences) was provided by Nathaniel Cannon and Dr. John Carlson (http://www.hardwoodgenomics.org/organism/*Castanea*/*mollissima*). Predicted genes were from regions of the genome with large numbers of SNPs associated with blight resistance/susceptibility (LaBonte et al., in preparation). These included a predicted MLP-like protein on linkage group (LG) C, two WD40 domain-containing proteins on LGF (g1803 and g1804), a sugar-transporter-like gene on LGF (g2785), a nicotinamidase-like gene on LGG, and an NBS-LRR-like gene on LGL. SNPs within these genes were identified that had one allele in all 16 from the blight association whole-genome resequencing project and a second allele fixed in the two American chestnut (*Cd*) genomes sequenced for that project. SNPs must be fixed for different alleles in *Cd* and *Cm* to be useful for marker-assisted selection in the backcross breeding program, so that the *Cm* alleles associated with resistance can be identified in the *Cm* background of advanced backcross trees. Additionally, based on the whole-genome sequence of 'Clapper,' primers were only designed for SNP loci with one *Cm* and one *Cd* allele in 'Clapper.' 'Clapper' is the main resistance donor for backcross breeding programs in Indiana and several other states.

Genotyping

A panel of trees was selected as a preliminary test of marker utility. BC3F1 trees with variable resistance to chestnut blight were selected from the Indiana TACF's germplasm collection. Two *Cd*, one *Cm*, and one F1 hybrid were included as controls. The goal of genotyping was to identify whether resistant BC3 trees contained more *Cm* alleles (*Cd/Cm* genotypes) at blight resistance SNPs than susceptible BC3 trees, which are expected to have more Cd alleles (*Cd/Cd* genotypes).

PCR protocols for the SNP markers were optimized in the lab by Aziz Ebrahimi. Once PCR was completed, PCR products were pooled by individual sample (i.e., PCR products for all ten loci were combined for each individual tree) and submitted to the Purdue Genomics Core Facility for WideSeq sequencing (https://www.purdue.edu/hla/sites/genomics/wideseq-2/). WideSeq is a cost-effective genotyping method because unlabelled primers can be used, and 10 or more markers can be multiplexed in one run, resulting in a final cost of about $2/sample/marker. WideSeq results consisted of thousands of short reads, which were assembled to the reference flanking sequence for each SNP marker. Assemblies were visualized using Geneious software (http://www.geneious.com, Kearse et al. 2012). Geneious was also used to identify SNPs and determine allele frequencies.

We screened additional trees in the IN-TACF program and TACF selections from Meadowview, VA, to further validate and optimize these SNPs. A larger population of BC3F1, additional BC3F2, and BC3F3 progeny was screened. Also, we tested marker inheritance for F1 (both [C × A] and [A × C] and BC1F1 trees in Indiana. Meadowview, VA selections included a range of BC3s with varied blight resistance. Novel *Cm* and *Cd* will were included as controls.

Plant Material Screened and Blight Disease Phenotypes

American, Chinese, F1, highly resistant and highly susceptible BC3 were used as species and known hybrid control genotypes (**Table 1A**). The BC1F1 seedlings were derived from our F1 selection (Line 4A) from our BC3F1 breeding block at Purdue University. American, Chinese, and F1 reference sources were provided from the IN-TACF and HTIRC. Meadowview VA resistant and susceptible BC3 selections were from eight to ten years old and resistant selections had maintained their original inoculated stem and survived natural blight pressure for two or more years (**Table 1B**). Cankers were measured and analyzed to calculate breeding values for resistance that we ranked from high to low. Other TACF B3F3 families from Meadowview, VA orchards were evaluated and tested (**Table 1C**). These families were grown by the IN-DNR Division of Forestry Vallonia Nursery in 2012/13, and planted as a progeny test at the Indiana Jackson Washington State Forest (JWSF) in 2014. In the winters of 2016 / 2017 and again in 2017 / 2018, all progeny were measured, evaluated for disease and scored on a "blight severity index" based on the percentage of progeny per family blighted added to an average canker severity among cankered progeny. For example, the worst family would score 3.0 because all progeny were blighted (1.0 or 100%), and they all scored (2.0) because every tree had large

cankers. Theoretically, a family with no blight would score 0. Our lowest ranked resistant family had 22% blight and an average severity of 0.25 for a BSI = 0.22 + 0.25 = 0.47.

In June of 2017, we inoculated all 2015 BC1F1 seedlings at SIPAC that had a 20 mm ground-line caliper or more, with the *C. parasitica* isolate SG, isolated from a blighted tree the IN-TACF Potawatomie Wildlife Park BC3F2 orchard in Tippecanoe, Indiana in early May, 2018. Cankers were visually evaluated that winter and 3 trees with the smallest cankers were collected and identified as "R" while 3 other trees with large cankers were included as "S" in February 2018.

Grafting Methods

Scion wood was received from the University of Tennessee, Chattanooga and the TN-TACF with new wild accessions of American trees from Alabama and Tennessee. BC3 scionwood was collected from the eight most disease-free individuals and families from a 2014 BC3F3 progeny test grown on the Jackson Washington State Forest in Indiana. Additional scionwood was collected from Duke American clones and seedlings in danger of removal due to blight at the Purdue FNR Martell Forest.

For the AL and TN American clones (30 in total), 16 were grafted onto four or five American 1-0 seedlings from the Vallonia and Hensler Nurseries. Up to six grafts of each JWSF resistant BC3 selection were grafted onto three BC3 and three BC1F1 1-0 seedlings. All rootstocks stocks were potted into 10 L tree pots with Metro Mix 560 / coir soilless potting mix in mid-April 2018. The trees were grown at the J.S. Wright Center greenhouse at Martell for 3 to 4 weeks before whip grafting. Whip grafts were made by hand and secured with budding rubbers, then coated with tree seal, and lastly covered with flat white interior latex paint. Trees were watered by hand with acidified complete nutrient solution (Peters 20-20-20).

In the field four-year-old American seedlings were cut at about five feet off the ground and one or two scions were bark grafted the first week of June, 2018 (Hartmann and Kester, 2011). Scion wood consisted of those accessions with limited wood that were not represented in the greenhouse study. One or two grafts per clone (14 clones in total), with one or two scions depending on the diameter of the rootstock, were bark grafted onto 19 trees. These American seedlings varied from 1.5 to 3-inches in diameter and were growing well with just the first blight arriving in 2017. Since 2017 and now into 2019, we have been treating cankers with fungicidal paint; (Propiconazole) [13 mL/gallon] mixed into flat white interior latex paint with 2% DMSO. Once grafted, all field graft unions were painted with this.

Successful American and BC3 greenhouse grafts grown outside in a shade house (30-50% shadecloth) and then were painted with the same fungicidal paint in late November before cold storage. In May 2019, the Americans were planted at the Duke Block at the Martell Forest while the BC3's were planted at our B3F2 orchard at Southern Indiana Purdue Agricultural Center (SIPAC) near Dubois, Indiana.

RESULTS

Genotyping

Linear regression of *Cm* allele percentage by field Canker Rating showed a highly significant correlation ($R^2 \leq 0.84$) between the two traits (**Figure 1**). Additional sites were identified in the genome where resistant BC3F3 pools had much higher frequencies of *Cm* alleles than susceptible pools (**Figure 2**). In the case of the disease-resistance-like locus on LGL, one of these segregating regions closely overlapped a candidate gene we targeted with a PCR-based SNP marker (lgl.8953a, b).

Genotypes were obtained in most individuals from seven SNP loci. Of the remaining three loci, one assembled poorly to the reference sequence, one was overly polymorphic, and one was monomorphic. The SNP loci lgf:g2785 and lgc:g3384 exhibited allele frequencies for hybrid genotypes ranging from approximately 0.56-0.75 (**Table 2A**). Most loci showed allele frequencies closer to the expected 0.5 for hybrid genotypes, 0.00 for *Cm* and 1.00 for *Cd* samples (**Table 2A**). Two *Cm*/*Cm* genotypes were observed in one of the resistant JWSF BC3F1 samples (**Sample 9; Table 2A**), indicating that this tree may result from a BC2 x BC2 cross rather than the BC2 x American cross used to generate a BC3F1, which is doubtful since this was a cross of a proven wild Indiana American ('Burke') from a woodlot near Martinsville, IN with pollen from Meadowview ('AB185'). More likely may be an error in sequencing/scoring which has to be managed in the lab with running known references and duplicate and often repeat sequencing runs. **Table 2B** designates the Indiana *Cd* mothers, TACF BC2 pollen parents, canker ratings, and estimated inoculated stem lifespan. For a BC3F1 tree, the maximum number of *Cm* alleles is equal to the number of loci: one *Cm* allele and one *Cd* allele at each locus. *Cm* allele numbers were tallied across SNP loci for BC3F1 individuals using: (*Cm* allele number / number of genotyped loci).

Allele frequencies easily conform to *Cd*/*Cd*, *Cd*/*Cm*, and *Cm*/*Cm* genotypes for novel Chinese, American, and new F1 crosses (**Table 3**). *Cd*/*Cd* is expected in susceptible BC3F1s while *Cd*/*Cm* is expected in resistant BC3F1s. The samples tested here include B1F1's that were inoculated and the 3 most resistant and three least resistant by a short-term rating of cankers didn't provide much difference in SNP profiles. We also included our three best 2nd generation seedlings, BC3F2's from our 1st F2 line that were inoculated at SIPAC in 2013, all of which scored fairly well and which had twice the number of *Cm* alleles compared to our B3F1 parents RL2 x GL367 and DOE x CH526. The F3's from Parke County, IN were planted as a memorial to Bill Craycraft who provided us years of seed and crossing with his Bloomingdale, IN trees. These represented three extra trees that had lost their tags while sorting our TACF B3 test for the Hoosier National Forest in 2012. It's likely that the third tree is a pure American and the other two are in fact hybrids.

Our largest set of samples to screen are presented in **Tables 4A-D**. Note that here, we changed our data to consider the proportion of *Cd* alleles rather than the proportion of *Cm* alleles as presented in Tables 2 and 3 previously. Unfortunately, the samples tested in all of these tables were combined into a 96 well plate and sequenced by the Purdue core genotyping facility and

whether sequencing errors occurred or PCR reactions were off, we found that repeat samples and known reference samples produced different results.

Table 4A shows a broader range of Chinese, American, and new F1samples and more SNP markers. Fortunately, we repeated the same DNA extracted for the 17.2 F1 hybrids from Table 3 and this repeat test shows two of these seedlings now scoring as *Cd / Cd* at a few loci. Our oldest F1 hybrid was an accidental natural Chinese x American hybrid (Line 4A) which is male sterile and confirmed by Sara Fitzsimmons years ago, ran as expected but several new Chinese sources scored as if they were hybrids, again underscoring the importance of repeat testing and using well established reference samples. In this test, we repeated the six B1F1 progeny from SIPAC and found similar results as previously where there was no difference and even slightly better scores for the "susceptible" versus "resistant" individuals (**Table 4B**). Unlike the previous test, our three most resistant B3F2's did not differentiate from their three susceptible progeny. Of concern is the nearly "perfect" score for our B3F2 open pollinated seedling (IN-TACF Line 2 father [Roselawn IN - 3 x GR97]). While that seedling has remained healthy and vigorous, clearly these results are incorrect.

The next group of samples in **Table 4C** represent the most resistant and susceptible eight families from our 2014 JWSF progeny test. Here again, we found little difference between groups despite the very big difference in disease between them (**Figure 3**). One seedling from the most resistant family (R1-B) showed a homozygous locus at SNP LGI_g330 3 but given the problems with this whole series of samples, we will need to re-test this tree to be sure. Other clear differences were observed in the growth rate of the "most resistant" or healthiest families compared to the most susceptible. Healthy trees after four-years with little blight or a few small cankers were noticeably smaller in height and diameter (**Figure 4**).

Table 4D, like the previous, compares eight resistant B3F3 individuals from TACF with 10 proven susceptible B3F3's. As with the younger Indiana material, SNP LGI_g330 3 showed that two resistant selections were homozygous for *Cm* alleles, and furthermore, that as two groups, they differed very little in the sum of *Cm* alleles (presented as mentioned as the converse of *Cd* alleles).

To overcome the problems of the previous 96 sample test, we decided to conduct an additional test where we pooled 1 to 10 trees of samples from related groups (e.g. resistant and susceptible trees), and illumina sequenced them, to now test the best 11 SNP's and another four that looked promising (15 SNP's in total). By doing this "genotyping through sequencing" approach, we probed the entire genome for the occurrence of these *Cm* and corresponding *Cd* alleles and were able to rectify the problems encountered with our previous test. **Table 5** shows the expected 1.00 , 0.50, 0.00 result for *Cd* alleles from American, F1, and Chinese samples respectively along with the number of individuals pooled and sequenced together. Here, we were also able to get a very clean and clear test of the most resistant long-term (16-year live stem) IN-TACF selection, our JWSF 3A tree (Burke X AB185) as a single tree pool by itself and as part of a broad pool combined with 9 other trees. This data clearly shows that a significant level of resistance is present with the TACF Meadowview, VA "R" vs. "S" scoring 0.69 and 0.93 respectively. Much

less difference was found for the Indiana B3F3 "R" vs. "S families" (0.90 vs. 0.93 respectively). The JWSF 3A tree by itself scored 0.78 and when it was combined in the pool with 9 others, that pool still scored close at 0.84.

Finally, **Figure 5** illustrates the full genome sequence we generated combining all the hybrid chestnuts we sampled. This stream of genomic data is not contiguous in correct chromosome order, but it provides a visual image of the entirety and complexity of how these alleles are distributed throughout the hybrid chestnut genome. Genomic regions for further exploration of informative SNP's are highlighted along with areas to avoid.

Grafting Results

Percent take was scored six to eight weeks after grafting. Greenhouse grafting of American clones faired much poorer than field grafting (**Table 6**). We didn't see any significant difference between BC3 and BC1 rootstock on the graft take of BC3 scions. We were disappointed that some clones failed to graft at all in the greenhouse (**Table 7**). We found much better graft-take in the field bark grafting the 14 clones with the most limited scion wood onto four-year American seedlings (**Table 8**).

DISCUSSION

SNP Markers for Blight Resistance

The predictive power of these SNP markers has been demonstrated across a wide genetic range of backcrossed hybrid chestnut families and generations. We will share these SNPs with all TACF chapters and other chestnut breeders to hopefully speed up chestnut blight resistance breeding. These markers should help many chapters and other breeders to classify their material. Given the clear discrimination between Chinese, American, and F1 trees, these blight resistant SNP's can confirm the pedigree of such trees when there is doubt. For those who need to distinguish an advanced hybrid such as a BC3 or BC4 from a pure American, these markers too will provide genetic proof of hybridization if even just one *Cm* allele is detected, and for those creating new F1 crosses, either with American or Chinese mother trees, these markers have proven robust on both types of F1 and every novel Chinese we have tested so far.

We found a few cases of *Cm / Cm* homozygous loci which would be desired in resistant parents to breed "true" for blight resistance. We found discrepancies with some loci for some samples during different lab test and suggest good known standards and doubling some samples to provide a replicated check to confirm the accuracy of all homozygous loci in particular and the overall SNP profile scores in general. Our first challenge is to find more hybrids with as many of these alleles as possible. For future hybrid chestnut breeding, parents with complimentary SNP blight profiles at these 15 different loci could help stack more blight resistance into new crosses.

As those few BC3's with moderate resistance emerge in the field, trees whose inoculated stems have survived for a decade or more can be grafted into new breeding orchards, open or cross pollinated, and then progeny can be screened with these SNP's in year-one to 1) evaluate the parents as good or poor resistance donors and 2) to rouge out susceptible seedlings and concentrate the most resistant into smaller more effective breeding blocks. Using pooled-sample sequencing, we found that a single resistant individual in a pool of ten trees will be detected, for example our JWSF 3A tree alone and in a pool of 9 others (**Table 5**). This sequencing approach also identified additional regions of the hybrid chestnut genome associated with differences in blight resistance (**Figure 5**). These regions should provide a source for more targeted SNP markers (for use in MAS) and/or candidate genes for blight resistance. The results of this research extends and enhances the products of a previous 2015 TACF grant, *"Assessing the functional genetic diversity of blight resistance in Chinese chestnut (Castanea mollissima Blume) by whole-genome resequencing of a diverse germplasm collection,"* and the combined results will appear within the next year in a peer-reviewed journal (LaBonte et al., 2020 - in preparation).

Grafting Discussion

The poor graft-take we had with our American clones in the greenhouse versus field bark grafting indicates we had poor American rootstocks. We had a lot of predation and disturbance of our 2016 American seed in both nurseries and a very wet 2017 spring that increased weeds and reduced seedling caliper. We struggled to get enough rootstocks large enough to match the good Alabama and Tennessee scion wood we had.

Our BC3 rootstock on the other hand fared much better and the graft-take was comparable to the field grafting, and previous results we have had at the HTIRC grafting chestnut. Our BC3 seed was sown in a richer area at the Vallonia nursery and the scionwood was very healthy and juvenile since it was collected from young four-year old trees. The better graft take in the field is likely due to much better vigor and health of the established trees. Such effects of rootstock health and quality are well known in horticulture.

While there are certainly practical and technical challenges to successfully graft American and hybrid chestnut, they can be met mostly with good horticultural practices, e.g., healthy rootstocks, healthy scion wood, good storage and growing conditions, etc. However, keeping ahead of chestnut blight is the biggest challenge that must be managed and considered. Grafting is a major wounding event and field grafting occurs in the late spring just when blight is very active and infectious. We bleached all scion wood as we brought it out of refrigeration and transported it to the JL Block in an ice-packed cooler the day of grafting. As we cut rootstocks, we sprayed all cut surfaces with 70% ethanol ahead of making each graft. Finally, after the graft was made and the sterile grafting wax was dry, we painted over the entire cut surface and scion wood to seal it all under a latex cover that we hoped would prevent new infection. It worked fairly well through the 2018 season but we have lost some trees in 2019. Finally, if the grafts take and the blight is avoided, the final challenge is delayed graft incompatibility. In a previous study, about 1/4 of the American chestnut clones grafted in our Duke orchard had failed by five

years (McKenna and Beheler, 2016). We hope that the IN-TACF and HTIRC will remeasure the Duke American clones this winter (2019-'20) to document how many grafts have persisted now to 10-years and to assess how many we have lost to chestnut blight over the last five-years.

We were able to expand our Duke American Orchard at the Martell Forest near Purdue and now have added the grafted American trees from Alabama and Tennessee (15 total in 2019), complimenting another row of a few Illinois seedlings planted in 2015, flanking our core Indiana American breeding orchard. We have maintained the 2015 JL Bark Graft American planting to continue to field test bark grafting and continue growing the successful original scions. We planted upwards of 5 grafts per clone of the "resistant" BC3 selections in each of the five random blocks we had reserved for eight new BC3F2 seedling lines. In total, we added 11 new grafted clones and left room to add another batch of selections for the future. SIPAC, and our Duke American orchard at Martell, are now functioning as two major germplasm conservation blocks for IN-TACF. We will provide a full report of the fate of these American and the SIPAC BC3 grafts at our winter meeting in 2020.

Certainly, the best means of controlling chestnut blight is to keep it out of the orchard. We are pleased that the IN-TACF has initiated a number of small American plantings over the last decade or so and many of their members are interested to help perpetuate the species by planting new clean blocks realizing that they may stay healthy for only five to 10-years. With a little planning and concerted effort, we can hopefully stay ahead of blight and conserve this valuable species to support future advanced chestnut breeding and conservation.

ACKNOWLEDGEMENTS

IN-TACF: Bruce Wakeland, Melanie Mount, Bill Deeter, Glenn Kotnik, Claude Diehl, and Ron Doyle; **Hensler Nursery:** Hamlet, Indiana; **Potawatomie Wildlife Park**: Tippecanoe, Indiana; **IN-DNR DoF:** Phil O'Connor, Bob Hawkins, Tyler Morlock, and Rob Wink; **University of Tennessee**: Hill Craddock and Trent Deason; **Purdue University – FNR:** Ron Rathfon, Lauren Mack, Daniel Mount, Brian Beheler, Michael Loesch-Fries, Marcia Kamer, Theresa Baker, Caleb Kell, and Matt Ginzel; **TACF:** Eric Evans, Greg Miller, and Sara Fitzsimmons; **Potawatomi Nature Park**: Tippecanoe, IN; **USDA-Forest Service**: Richard Sniezko and Jennifer Koch.

Funding: was provided by a 2017 TACF Grant with additional financial support provided by the Fred M. van Eck Foundation of the Hardwood Tree Improvement and Regeneration Center, Forestry & Natural Resources, Purdue University, and the USDA Forest Service, Northern Research Station.

REFERENCES

- Anagnostakis SL (1987) Chestnut blight: the classical problem of an introduced pathogen. Mycologia 79(1):23-27.
- Bortesi L, Fischer R (2015) The CRIPSR/Cas9 system for plant genome editing and beyond. Biotechnol Adv 33(1):41-52.
- Barakat A, DiLoreto, DS, Zhang Y, Smith C, Baier K, Powell WA, Wheeler N, Sederoff R, Carlson JE (2009) Comparison of the transcriptomes of American chestnut (*Castanea dentata*) and Chinese chestnut (*C. mollissima*) in response to the chestnut blight infection. BMC Plant Biology 9:51.
- Barakat A, Staton M, Cheng C-H, et al. (2012) Chestnut resistance to the blight disease: insights from transcriptome analysis. BMC Plant Biology 12:38.
- Burnham CR, Rutter PA, French DW (1986) Breeding blight-resistant chestnuts. Plant Breeding Reviews 4:347-397.
- Collard, B.C., and Mackill, D.J. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philos Trans R Soc Lond B Biol Sci*. 2008; 363(1491):557–572. doi:10.1098/rstb.2007.2170
- Fan D, Liu TT, Li CF, Jiao B, Li S, Hou YS, Luo KM (2015) Efficient CRISPR/Cas9-mediated targeted mutagenesis in *Populus* in the first generation. Sci Rep 10.1038/srep12217.
- Fang G-C, Blackmon BP, Staton ME, Nelson CD, Kubisiak TL, et al. (2012) A physical map of the Chinese chestnut genome and its integration with the genetic map. Tree Genetics and Genomes 9(2):525-537.
- Hartmann and Kester's plant propagation (2011): principles and practice / Fred T. Davies, Robert L. Geneve, Dale E. Kester. 8th ed.
- Hebard FV, Griffin GJ, Elkins JR (1984) Developmental histopathology of canker incited by hypovirulent and virulent isolates of *Endothia parasitica* on susceptible and resistance chestnut trees. Phytopathology 74:140-149.
- Jia JG, Wang N (2014) Targeted genome editing of sweet orange using Casi/sgRNA. PloS One 9: e93806.
- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P., and Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*(12), 1647-1649.
- Kubisiak TL, Hebard FV, Nelson CD, Zhang J, Bernatzky R, Huang J, Anagnostakis SL, Doudrick RL (1997) Molecular mapping of resistance to blight in an interspecific cross in the genus *Castanea*. Phytopathology 87:751-759.
- Kubisiak TL, Nelson CD, Staton ME, Zhebentyayeva T, Smith C, Olukolu BA, Fang G- C, Hebard FV, Anagnostakis S, Wheeler N, Sisco PH, Abbott AG, Sederoff RR (2013) A transcriptome-based genetic map of Chinese chestnut (Castanea mollissima) and identification of regions of segmental homology with peach (Prunus persica). Tree Genetics and Genomes 9:557-571.
- LaBonte, N., Miller, G., Nelson, C.D., Woeste, K. (2019 *in preparation*) Genetic variants associated with blight resistance and signatures of selection near blight resistance loci.
- McKenna, J. and Beheler B. (2016) Five-year graft compatibility in an American chestnut breeding orchard. *Journal of the American Chestnut Foundation*. Winter Edition. p. 24-28.
- Powell WA, Morley P, King M, Maynard CA (2007) Small stem chestnut blight resistance assay. Journal of the American Chestnut Foundation 21(2):34-38.

- Serrazina SMT, Santos C, Machado H, Pesquita C, Vicentini R, Pais M, Sebastiana M, Costa RL (2015) Castanea root transcriptome in response to Phytophthora cinnamomi challenge. Tree Genetics and Genomes 11(1) DOI: 10.1007/s11295-014-0829-7
- Staton ME, Zhebentyayeva T, Olukolu B, Fang GC, Nelson D, Carlson JE, Abbott AG (2015) Substantial genome synteny preservation among woody angiosperm species: comparative genomics of Chinese chestnut (*Castanea mollissima*) and plant reference genomes. BMC Genomics 16:744.
- Tsai C-J, Xue L-J (2015) CRISPRing into the woods. GM Crops Food 6(4):206-215.
- Zhang B, Newhouse A, McGuigan L, Maynard C, Powell W (2011) Agrobacterium-mediated co-transformation of American Chestnut (*Castanea dentata*) somatic embryos with a wheat oxalate oxidase gene. BMC Proc 5(Suppl 7):O43.
- Stanke, M., Schöffman, O., Morgenstern, B., Waack, S. (2006) Gene prediction in eukaryotes with a generalized hidden Markov model that uses hints from external sources. *BMC Bioinformatics* 7, 62.

FIGURES AND TABLES

Table 1A. American, Chinese, and F1 hybrid chestnut plant material screened and their resistance and susceptibility phenotype designations. Average blight %, where available, was based on the presence or absence of the disease on all progeny per family after four years in the field under high blight pressure at the Jackson Washington State Forest (JWSF), near Salem, IN.

IN-TACF - HTIRC Identity	TACF Accesion No.	Spp. / Genotype	R/S Fam.	Avg. Blight (%)	Provider / Notes
Martell Chin x Am 'Johnson'	-	F1	R	-	Cross C x A 2016 / '17 Val
Martell Chin x Am 'Johnson'	-	F1	R	-	Cross C x A 2016 / '17 Val
Martell Chin x Am 'Johnson'	-	F1	R	-	Cross C x A 2016 / '17 Val
14-137 (outstanding form-1)	D5-18-101	B3F3	MR	57%	JWSF 2014 TACF B3F3 - Excellent AC Form
14-137 (outstanding form-2)	D5-18-101	B3F3	MR	"	JWSF 2014 TACF B3F3 - Excellent AC Form
14-137 (outstanding form-3)	D5-18-101	B3F3	MR	"	JWSF 2014 TACF B3F3 - Excellent AC Form
LINE 2 IN-TACF R4-T17	IN AC RL3 x TACF GR97	B3F2	R	36%	JWSF 2014 TACF B3F3
CH88 (BC3F2)	CH88 (BC3F2)	B3F2	R	33%	TACF Meadowview, VA
14-156 (Greg Miller Missouri)	MO- Chinese G. Miller	CC	R++	7%	JWSF 2014 TACF B3F3
14-157 (Greg Miller Ohio)	OH- Chinese G. Miller	CC	R++	9%	JWSF 2014 TACF B3F3
14-158 (Wilkinson Chin.)	Wilkinson Chinese	CC	R++	1%	JWSF 2014 TACF B3F3
Hort Chin sdlg	-	CC	R++	0%	Lugar Farm Chinese Orch / Val '17
Hort Chin sdlg	-	CC	R++	0%	Lugar Farm Chinese Orch / Val '17
Hort Chin sdlg	-	CC	R++	0%	Lugar Farm Chinese Orch / Val '17
LINE 3A	IN AC Burke x TACF AB185	B3F1	R+	-	JWSF 2003 - IN-TACF best tree
Duke Am sdlg	-	AC	S	-	Duke Orch / Val '17
Duke Am sdlg	-	AC	S	-	Duke Orch / Val '17
Duke Am sdlg	-	AC	S	-	Duke Orch / Val '17
14-150	MGL Mix	AC	S	75%	TACF Meadowview, VA
14-154	HARLAN CO. AM. MIX	AC	S	88%	TACF Meadowview, VA
Bloomingdale East - Green	-	AC	S	75%	Friends Church
Bloomingdale West - White	-	AC	S	91%	Friends Church

Table 1B. Resistant and susceptible individuals growing at Meadowview, VA screened with SNP resistance markers. Resistant selections were older than 11-years in the field and had been inoculated 2 or more years prior to measuring canker sizes and calculating predicted breeding values based on best-linear-unbiased-predictors (BLUP's). For resistant individuals, with the highest BLUP, R1 had the smallest cankers and R8 had the largest in this resistance class; for the susceptible class, S1 had the lowest BLUP and thus the largest cankers relative to S8.

TACF Accesion No.	Spp. / Genotype	R/S / Ind.	**BLUP**	Provider / Notes
D6-27-20	B3F3 (Clapper)	R1	**53.7**	TACF Meadowview, VA
D1-27-54	B3F3 (Clapper)	R2	**51.8**	TACF Meadowview, VA
D3-17-73	B3F3 (Clapper)	R3	**51.0**	TACF Meadowview, VA
D5-20-15	B3F3 (Clapper)	R4	**47.6**	TACF Meadowview, VA
D4-9-105	B3F3 (Clapper)	R5	**45.7**	TACF Meadowview, VA
D3-18-61	B3F3 (Clapper)	R6	**44.7**	TACF Meadowview, VA
D6-27-4	B3F3 (Clapper)	R7	**43.4**	TACF Meadowview, VA
D1-17-99	B3F3 (Clapper)	R8	**38.2**	TACF Meadowview, VA
D5-26-54	B3F3 (Clapper)	S1	**-13.2**	TACF Meadowview, VA
D5-29-124	B3F3 (Clapper)	S2	**-11.6**	TACF Meadowview, VA
D2-29-122	B3F3 (Clapper)	S3	**-3.8**	TACF Meadowview, VA
D5-1-4	B3F3 (Clapper)	S4	**-3.7**	TACF Meadowview, VA
D4-26-43	B3F3 (Clapper)	S5	**-0.3**	TACF Meadowview, VA
D4-11-98	B3F3 (Clapper)	S6	**0.3**	TACF Meadowview, VA
D4-12-29	B3F3 (Clapper)	S7	**1.5**	TACF Meadowview, VA
D4-29-72	B3F3 (Clapper)	S8	**4.4**	TACF Meadowview, VA
D4-17-59	B3F3 (Clapper)	S9	**5.2**	TACF Meadowview, VA
D2-10-18	B3F3 (Clapper)	S10	**6.5**	TACF Meadowview, VA

Table 1C. Resistant and susceptible individuals growing at JWSF, Salem, IN, planted adjacent to a 15-year-old B3F1 block where blight is endemic, screened with SNP resistance markers. All trees were evaluated for chestnut blight after four years and the average of all progeny per family is presented. R1-A represents the best seedling from the best family. R8-B is thus the 2nd best progeny from the "eighth most resistant family." For the susceptible class only two seedlings were sampled; S1-A had the worst disease incidence S8-B had the "best" of the susceptible class.

IN-TACF - HTIRC Identity	TACF Accesion No.	Spp. / Genotype	R/S Fam.	Avg. Blight (%)	Provider / Notes
14-125	D3-29-1	B3F3	R1-A	22%	JWSF 2014 TACF B3F3
14-125	D3-29-1	B3F3	R1-B	"	JWSF 2014 TACF B3F3
14-125	D3-29-1	B3F3	R1-C	"	JWSF 2014 TACF B3F3
14-125	D3-29-1	B3F3	R1-D	"	JWSF 2014 TACF B3F3
14-125	D3-29-1	B3F3	R1-E	"	JWSF 2014 TACF B3F3
14-125	D3-29-1	B3F3	R1-F	"	JWSF 2014 TACF B3F3
14-104	D1-17-4	B3F3	R2-A	38%	JWSF 2014 TACF B3F3
14-104	D1-17-4	B3F3	R2-B	"	JWSF 2014 TACF B3F3
14-104	D1-17-4	B3F3	R2-C	"	JWSF 2014 TACF B3F3
14-129	D4-20-65	B3F3	R4-A	33%	JWSF 2014 TACF B3F3
14-129	D4-20-65	B3F3	R4-B	"	JWSF 2014 TACF B3F3
14-105	D1-21-25	B3F3	R5-A	40%	JWSF 2014 TACF B3F3
14-105	D1-21-25	B3F3	R5-B	"	JWSF 2014 TACF B3F3
14-142	D6-26-27	B3F3	R6-A	30%	JWSF 2014 TACF B3F3
14-142	D6-26-27	B3F3	R6-B	"	JWSF 2014 TACF B3F3
14-142	D6-26-27	B3F3	R6-C	"	JWSF 2014 TACF B3F3
14-113	D1-28-19	B3F3	R7-A	33%	JWSF 2014 TACF B3F3
14-113	D1-28-19	B3F3	R7-B	"	JWSF 2014 TACF B3F3
14-113	D1-28-19	B3F3	R7-C	"	JWSF 2014 TACF B3F3
14-107	D1-26-105	B3F3	R8-A	38%	JWSF 2014 TACF B3F3
14-107	D1-26-105	B3F3	R8-B	"	JWSF 2014 TACF B3F3
14-111	D1-27-140	B3F3	S1-A	67%	JWSF 2014 TACF B3F3
14-111	D1-27-140	B3F3	S1-B	"	JWSF 2014 TACF B3F3
14-111	D1-27-140	B3F3	S1-C	"	JWSF 2014 TACF B3F3
14-145	D7-10-145	B3F3	S2-A	100%	JWSF 2014 TACF B3F3
14-145	D7-10-145	B3F3	S2-B	"	JWSF 2014 TACF B3F3
14-136	D5-1-76	B3F3	S3-A	50%	JWSF 2014 TACF B3F3
14-136	D5-1-76	B3F3	S3-B	"	JWSF 2014 TACF B3F3
14-119	D2-27-78	B3F3	S4-A	88%	JWSF 2014 TACF B3F3
14-119	D2-27-78	B3F3	S4-B	"	JWSF 2014 TACF B3F3
14-131	D4-22-103	B3F3	S5-A	80%	JWSF 2014 TACF B3F3
14-131	D4-22-103	B3F3	S5-B	"	JWSF 2014 TACF B3F3
14-110	D1-27-134	B3F3	S6-A	83%	JWSF 2014 TACF B3F3
14-110	D1-27-134	B3F3	S6-B	"	JWSF 2014 TACF B3F3
14-108	D1-26-37	B3F3	S7-A	83%	JWSF 2014 TACF B3F3
14-108	D1-26-37	B3F3	S7-B	"	JWSF 2014 TACF B3F3
14-149	D9-21-113	B3F3	S8-A	89%	JWSF 2014 TACF B3F3
14-149	D9-21-113	B3F3	S8-B	"	JWSF 2014 TACF B3F3

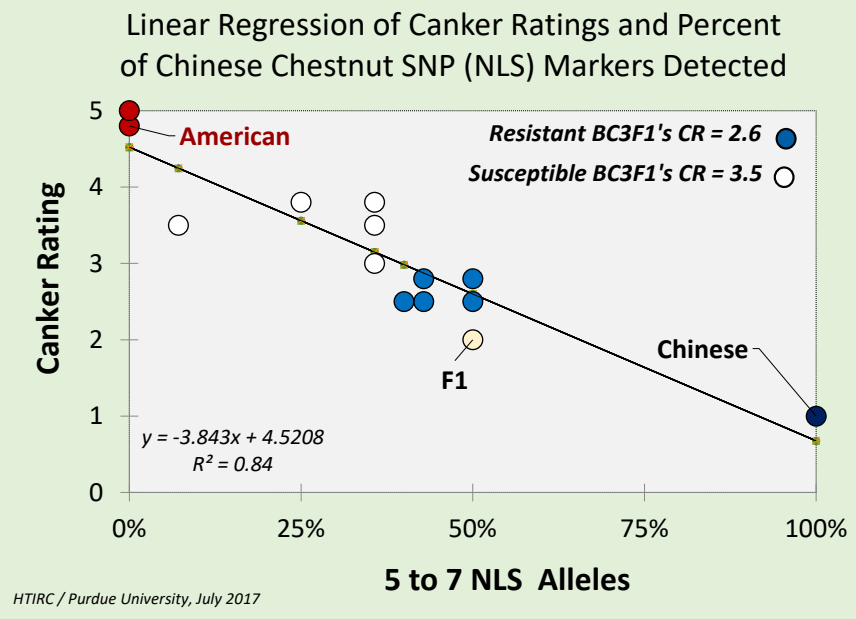

Figure 1. Correlation of NLS Markers with 6 to 16 year IN-TACF BC3F1 progeny grown in Starke, Tippecanoe, and Washington Counties of Indiana. The F1 hybrid is a novel natural cross of DOE x a Chinese chestnut in Grant Co., Indiana. The Chinese is 'Hort 14' that was purchased about 30 years ago from Empire Chestnut. American chestnuts were both grafted clones from the Duke Energy Orchard at Martell Forest, West Lafayette, IN.

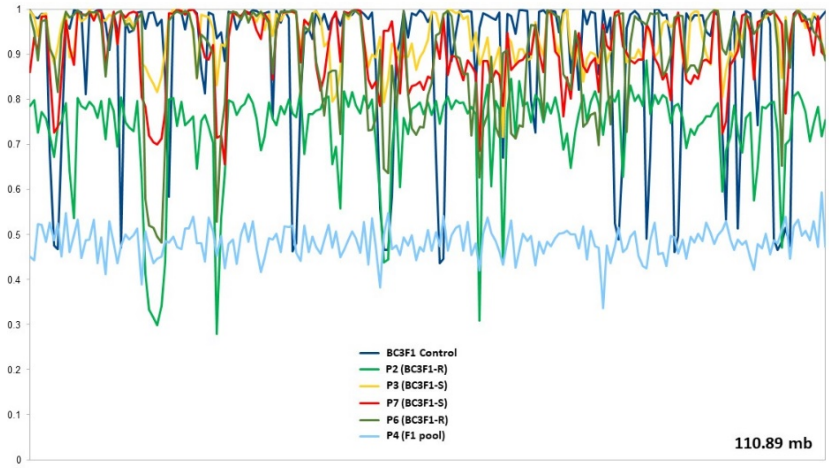

Figure 2. Allele frequencies of American chestnut alleles (Y axis) for all informative SNP loci on Linkage Group A of the *C. mollissima* reference genome. Resistant pools are in green; susceptible pools yellow and orange; F1 control pool is in light blue. Note that some BC3F3 pools show a greater proportion of Chinese chestnut alleles than F1's at some sites, indicating successful selection for *Cm/Cm* segregants at blight resistance loci.

Table 2A. Genotypes coded for species of origin (American chestnut allele = *Cd*, Chinese chestnut allele = *Cm*) at seven SNP loci designed from exons of blight-resistance candidate genes in a panel of BC3F1 chestnuts, American chestnuts (*Cd*), Chinese chestnut (*Cm*) and one F1 hybrid.

Lab no.	Species	Blight	lgc:g3384	lgf:g1803	lgf:g1804	lgf:g2785	lgg:g3657b	lgl_g8953a	lgl_g8953b
1	BC3F1	R	Cd/Cm	Cd/Cm	Cd/Cm	Cd/Cm	Cd/Cd	Cd/Cm	Cd/Cm
2	BC3F1	M	Cd/Cm	Cd/Cd	Cd/Cm	x	x	Cd/Cm	Cd/Cm
3	BC3F1	S	Cd/Cm	Cd/Cd	Cd/Cd	Cd/Cd	x	Cd/Cm	Cd/Cm
4	BC3F1	S	Cd/Cm	Cd/Cm	Cd/Cm	Cd/Cm	Cd/Cm	Cd/Cd	Cd/Cd
5	Chinese	R+	Cm/Cm	Cm/Cm	Cm/Cm	Cm/Cm	Cm/Cm	Cm/Cm	Cm/Cm
6	American	S+	Cd/Cd	Cd/Cd	Cd/Cd	Cd/Cd	Cd/Cd	Cd/Cd	Cd/Cd
7	American	S+	Cd/Cd	Cd/Cd	Cd/Cd	Cd/Cd	Cd/Cd	Cd/Cd	Cd/Cd
8	BC3F1	R+	x	x	Cd/Cm	Cd/Cm	Cd/Cm	Cd/Cm	Cd/Cm
9	BC3F1	R	Cm/Cm	Cd/Cm	Cd/Cm	Cd/Cd	Cm/Cm	Cd/Cd	Cd/Cd
10	BC3F1	M	Cd/Cm	Cd/Cm	Cd/Cm	Cd/Cd	Cd/Cd	Cd/Cm	Cd/Cm
11	BC3F1	S	Cd/Cd	Cd/Cd	Cd/Cd	Cd/Cd	Cd/Cm	Cd/Cd	Cd/Cd
12	BC3F1	R	Cd/Cm	Cd/Cm	Cd/Cm	Cd/Cd	Cd/Cd	Cd/Cm	Cd/Cm
13	BC3F1	M	Cd/Cm	Cd/Cm	Cd/Cm	Cd/Cm	Cd/Cm	Cd/Cm	Cd/Cm
14	F1	R	Cd/Cm	Cd/Cm	x	Cd/Cm	Cd/Cm	Cd/Cm	Cd/Cm

[a]R+ = highly resistant; R = resistant, M = moderately resistant, S = susceptible, S+ = highly susceptible

Table 2B. Summary of BC3F1 selections, F1, and chestnut species used to validate seven novel SNP loci.

Lab No.	Geno.	IN-TACF BC3F1 Selection	Blight Resist	Orchard	IN-TACF Line	IN *Cd* Mother	TACF BC2 Father	CR (2017)	Yrs. Live Stem	Loci Geno-typed	*Cm* alleles
1	BC3F1	Wake. A	R	Culver	1A	IW2	GR226	2.5	16	7	6
2	BC3F1	Wake. B	R	Culver	1B	IW2	GR226	2.5	14	5	4
3	BC3F1	Wake. C	S	Culver	1C	IW2	GR226	3.8	5	6	3
4	BC3F1	Wake. D	S	Culver	1D	IW2	BE325	3.5	7	7	5
5	Chinese	Hort #14	R+	Lugar	-	-	-	1.0	30	7	14
6	American	'Sieg #2'	S+	Duke	-	-	-	5.0	2	7	0
7	American	'Johnson'	S+	Duke	-	-	-	4.8	2	7	0
8	BC3F1	JWSF '3A'	R+	JWSF	3A	BURKE	AB185	2.5	14	5	5
9	BC3F1	JWSF '3B'	R	JWSF	3B	BURKE	AB185	2.8	14	7	6*
10	BC3F1	JWSF '3C'	M	JWSF	3C	BURKE	AB185	3.8	9	7	5
11	BC3F1	JWSF '3D'	S	JWSF	2E	RL3	GR97	3.5	6	7	1
12	BC3F1	JWSF '3E'	R	JWSF	2D	RL3	GR97	3.0	14	7	5
13	BC3F1	JWSF '3F'	R	JWSF	3D	BURKE	AB185	2.8	14	7	7
14	F1	Line 4A	R+	Lugar	4A	DOE	CHIN op	2.0	12	6	6

[a] R+ = highly resistant, R = resistant, M = moderately resistant, S = susceptible, S+ = highly susceptible, CR = canker rating, Years stem lived after inoculation, * two loci homozygous for *Cm* need to be investigated further.

Table 3. Seven of the best initial SNP's tested to further validate differential resistance.

						SNP Locus									
Sample ID	Spp. Genotype	Orchard	Block	R-T	CR*	Lgc_3384:b37	LGF_g1803:b89	LGF_g1804:b83	LGF_g2785:b104	LGG_g3657b:b35	LGL_g8953a:b109	LGL_g8953b:b125	*Cm* dosage	Loci	*Cm*/ locus
Sigma Chi	CC	Martell	na	na	R+	Cm	Cm	Cm	Cm	Cm	Cm	Cm	12	6	2.00
East barn	CC	SIPAC	na	na	R+	Cm	Cm	Cm	Cm	Cm	Cm	Cm	14	7	2.00
West barn	CC	SIPAC	na	na	R+	Cm	Cm	Cm	Cm	Cm	Cm	Cm	14	7	2.00
17-2.1	F1	Martell	na	na	R	--	Cm/Cd	Cm/Cd	--	Cm/Cd	Cm/Cd	Cm/Cd	5	5	1.00
17-2.2	F1	Martell	na	na	R	--	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	6	6	1.00
17-2.3	F1	Martell	na	na	R	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	7	7	1.00
17-4.1	AC	Duke	na	mix	S+	Cd	Cd	Cd	Cd	Cd	Cd	Cd	0	7	0.00
17-4.2	AC	Duke	na	mix	S+	Cd	Cd	Cd	Cd	Cd	Cd	Cd	0	7	0.00
17-4.3	AC	Duke	na	mix	S+	Cd	Cd	Cd	Cd	Cd	Cd	Cd	0	7	0.00
Line 4A	B1F1	SIPAC	1	R1-T17	"R"	--	Cd	Cd	Cm/Cd	Cd	Cd	Cd	1	6	0.17
Line 4A	B1F1	SIPAC	1	R1-T18	"R"	Cd	Cd	Cd	Cm/Cd	Cd	Cd	Cd	1	7	0.14
Line 4A	B1F1	SIPAC	1	R2-T20	"R"	--	Cm/Cd	Cm/Cd	Cd	--	Cm/Cd	Cm/Cd	3	5	0.60
Line 4A	B1F1	SIPAC	1	R2-T15	"S"	Cm/Cd	Cd	Cd	Cm/Cd	Cm/Cd	Cm	Cm	3	7	0.43
Line 4A	B1F1	SIPAC	1	R2-T10	"S"	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	Cd	Cd	Cd	4	7	0.57
Line 4A	B1F1	SIPAC	1	R2-T9	"S"	Cd	Cd	Cd	Cd	Cm	Cm/Cd	Cm/Cd	4	7	0.57
Line 1A	B3F2	SIPAC	1	R3-T10	R	Cm/Cd	Cd	Cd	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	5	7	0.71
Line 1A	B3F2	SIPAC	2	R6-T12	R	Cm/Cd	Cd	Cd	Cm/Cd	Cd	Cm/Cd	Cm/Cd	4	7	0.57
Line 1A	B3F2	SIPAC	1	R1-T20	R	Cm/Cd	--	Cm/Cd	Cm/Cd	Cd	Cm/Cd	Cm/Cd	5	6	0.83
RL2 x GL367	B3F1	Lugar	na	R21-T23	R	Cm/Cd	Cd	Cm/Cd	Cd	Cd	Cd	Cd	2	7	0.29
DOE x CH526	B3F1	Lugar	na	R1-T53	R	Cd	Cd	Cm/Cd	Cd	Cm/Cd	Cd	Cd	2	7	0.29
RL3 x GR97	B3F1	Lugar	na	R17-T4	R										
TACF '12-F3	B3F3	Parke Co.	ns	na	R?	Cm/Cd	Cm	Cd	Cd	Cd	Cd	Cd	3	7	0.43
TACF '12-F3	B3F3	Parke Co.	ns	na	R?	Cd	Cd	Cm/Cd	Cd	Cm/Cd	Cm/Cd	Cm/Cd	4	7	0.57
TACF '12-F3	B3F3	Parke Co.	ns	na	R?	Cd	Cd	Cd	Cd	Cd	Cd	Cd	0	7	0.00

*CR= Field canker rating			
	R+	Highly resistant	R?
	R	Resistant	"S"
	"R"	2-month SG canker was small	S+

Table 4A. American, F1, and Chinese samples for our third round of screening with 11 SSR's. Note this data based on *Cd* alleles present rather than *Cm*. Errors occurred which are highlighted.

HTIRC / TACF ID	Spp. / Genotype	Provider	R / S	LGF_g18 03		LGF_g18 04		LGA_g14 20		LGA_g84 59		LGA_g41 71		LGB_g22 12		LGD_g11 64		LGG_g62 52		LGI_g330 3		LGB_g10 84		LGG_g23 09		Total *Cd* Alleles	*Cd* Allelic Avg
Duke Am sdlg	AC	Duke Orch / Val '17	S	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	.	.	19	0.95
Duke Am sdlg	AC	Duke Orch / Val '17	S	1	1	0	1	0	1	0	1	0	1	0	1	.	.	1	1	1	1	1	1	.	.	13	0.81
Duke Am sdlg	AC	Duke Orch / Val '17	S	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	.	.	19	0.95
Duke Am sdlg	AC	Duke Orch / Val '16 / Pots '17	S	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	20	0.91
Bloomingdale West	AC	Friends Church	S	0	1	0	1	0	1	1	1	0	1	1	1	0	1	1	1	1	1	0	1	.	.	14	0.70
Bloomingdale East	AC	Friends Church	S	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	.	.	18	0.90
Martell Chin x Am 'Johnson' sdlg	F1	C x A 2016 / '17 Val	R	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	.	.	10	0.50
Martell Chin x Am 'Johnson' sdlg	F1	C x A 2016 / '17 Val	R	0	1	0	1	0	1	.	.	1	1	0	1	0	1	0	1	.	.	1	1	0	0	10	0.56
Martell Chin x Am 'Johnson' sdlg	F1	C x A 2016 / '17 Val	R	0	1	0	1	0	1	0	1	0	0	0	1	0	1	0	1	0	1	0	1	0	1	10	0.45
17-2.1	F1	A x C 2015 / '16 Val/ '17 pots	R	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	11	0.50
17-2.2	F1	A x C 2015 / '16 Val/ '17 pots	R	0	0	.	.	0	0	.	.	1	1	.	.	.	.	.	.	.	.	.	.	.	.	2	0.33
17-2.3	F1	A x C 2015 / '16 Val/ '17 pots	R	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	0	10	0.45
Line 4A mother	F1	Line 4A ortet, Lugar Farm	R+	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	11	0.50
14-156 (Greg Miller Missouri)	CC	JWSF 2014 TACF B3F3	R++	0	0	0	0	0	1	0	1	0	1	0	1	1	1	1	1	1	1	0	1	.	.	11	0.55
14-158 (Wilkinson Chin.) VA	CC	JWSF 2014 TACF B3F3	R++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	3	0.14
14-157 (Greg Miller Ohio)	CC	JWSF 2014 TACF B3F3	R++	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	.	.	18	0.90
Hort Chin sdlg	CC	Lugar Farm Chinese Orch / Val '17	R++	1	1	1	1	1	1	.	.	.	.	1	1	1	1	0	1	0	1	.	.	.	.	12	0.86
Hort Chin sdlg	CC	Lugar Farm Chinese Orch / Val '17	R++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	3	0.14
Hort Chin sdlg	CC	Lugar Chinese Val '16 / Pots '17	R++	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0.05
Martell Chinese	CC	Martell 2017 twig	R++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00
SIPAC East Chin	CC	SIPAC east of barn	R++	0	0	0	0	0	0	.	.	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0.05
SIPAC West Chin	CC	SIPAC west of barn	R++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0.05

Table 4B. B1, B3F1, and B3F2 samples split between "R" and "S" for our third round of screening with 11 SSR's. Note this data based on *Cd* alleles present rather than *Cm*. Errors occurred which are highlighted. Also, three outstanding American form trees were collected from family '14-137' aka TACF 'D5-18-101.'

HTIRC / TACF ID	Spp. /Gen	Provider	R / S	LGF_g18 03		LGF_g18 04		LGA_g14 20		LGA_g84 59		LGA_g41 71		LGB_g22 12		LGD_g11 64		LGG_g62 52		LGI_g330 3		LGB_g10 84		LGG_g23 09		Total *Cd* Alleles	*Cd* Allelic Avg
Line 4A op	B1F1	SIPAC IN-TACF	R1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	.	.	18	0.90
Line 4A op	B1F1	SIPAC IN-TACF	R2	1	1	0	1	0	1	0	1	0	1	0	1	1	1	0	1	1	1	0	1	0	1	14	0.64
Line 4A op	B1F1	SIPAC IN-TACF	R3	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	20	0.91
Line 4A op	B1F1	SIPAC IN-TACF	S1	1	1	0	1	0	1	0	1	0	1	0	1	0	1	1	1	1	1	1	1	0	1	15	0.68
Line 4A op	B1F1	SIPAC IN-TACF	S2	0	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	18	0.82
Line 4A op	B1F1	SIPAC IN-TACF	S3	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	19	0.86
LINE 3A	B3F1	JWSF 2003	R+																							0	
Line 2 Male Sdlg	B3F2	JWSF 2014 IN-TACF B3F2 o.p.	R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0.05
Line 1A	B3F2	SIPAC IN-TACF	R1	1	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	.	.	17	0.85
Line 1A	B3F2	SIPAC IN-TACF	R2																							0	
Line 1A	B3F2	SIPAC IN-TACF	R3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	21	0.95
Line 1A	B3F2	SIPAC IN-TACF	S1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	.	.	19	0.95
Line 1A	B3F2	SIPAC IN-TACF	S2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	.	.	19	0.95
Line 1A	B3F2	SIPAC IN-TACF	S3	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	.	.	16	0.80
14-137 (outstanding form)	B3F3	JWSF 2014 TACF B3F3	R																							0	
14-137 (outstanding form)	B3F3	JWSF 2014 TACF B3F3	R	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	21	0.95
14-137 (outstanding form)	B3F3	JWSF 2014 TACF B3F3	R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	21	0.95

Table 4C. Comparison of "R" and "S" families screened with 11 SSR's. Note this data based on *Cd* alleles present rather than *Cm*. Trees were infected beginning their first year. Selections were based on the eight least cankered families (see Table 1C for canker ratings based on family blight %), and the least cankered individuals per family compared to two seedlings from the most susceptible "S" families.

HTIRC / TACF ID	Spp. / Genotype	Provider	R / S	LGF_g18 03		LGF_g18 04		LGA_g14 20		LGA_g84 59		LGA_g41 71		LGB_g22 12		LGD_g11 64		LGG_g62 52		LGI_g330 3		LGB_g10 84		LGG_g23 09		Total *Cd* Alleles	*Cd* Allelic Avg
14-125	B3F3	JWSF 2014 TACF B3F3	R1-A	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	.	.	17	0.85
14-125	B3F3	JWSF 2014 TACF B3F3	R1-B	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	**0**	**0**	1	1	.	.	16	0.80
14-125	B3F3	JWSF 2014 TACF B3F3	R1-C	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	19	0.86
14-125	B3F3	JWSF 2014 TACF B3F3	R1-D	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	.	.	18	0.90
14-125	B3F3	JWSF 2014 TACF B3F3	R1-E	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	.	.	18	0.90
14-125	B3F3	JWSF 2014 TACF B3F3	R1-F	.	.	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	.	.	17	0.94
14-104	B3F3	JWSF 2014 TACF B3F3	R2-A	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	21	0.95
14-104	B3F3	JWSF 2014 TACF B3F3	R2-B	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	.	.	19	0.95
14-104	B3F3	JWSF 2014 TACF B3F3	R2-C	1	1	1	1	1	1	1	1	0	1	0	1	0	1	1	1	0	1	0	1	1	1	17	0.77
14-129	B3F3	JWSF 2014 TACF B3F3	R4-A	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0	1	1	1	19	0.86
14-129	B3F3	JWSF 2014 TACF B3F3	R4-B	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	.	.	17	0.85
14-105	B3F3	JWSF 2014 TACF B3F3	R5-A	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	.	.	18	0.90
14-105	B3F3	JWSF 2014 TACF B3F3	R5-B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	.	.	20	1.00
14-142	B3F3	JWSF 2014 TACF B3F3	R6-A	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	.	.	18	0.90
14-142	B3F3	JWSF 2014 TACF B3F3	R6-B	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	21	0.95
14-142	B3F3	JWSF 2014 TACF B3F3	R6-C	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	.	.	18	0.90
14-113	B3F3	JWSF 2014 TACF B3F3	R7-A	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	.	.	18	0.90
14-113	B3F3	JWSF 2014 TACF B3F3	R7-B	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	.	.	19	0.95
14-113	B3F3	JWSF 2014 TACF B3F3	R7-C	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	.	.	17	0.85
14-107	B3F3	JWSF 2014 TACF B3F3	R8-A	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	.	.	17	0.85
14-107	B3F3	JWSF 2014 TACF B3F3	R8-B	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	19	0.86
14-111	B3F3	JWSF 2014 TACF B3F3	S1-A	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	.	.	19	0.95
14-111	B3F3	JWSF 2014 TACF B3F3	S1-B	.	.	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	.	.	17	0.94
14-111	B3F3	JWSF 2014 TACF B3F3	S1-C	1	1	0	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	.	.	17	0.85
14-145	B3F3	JWSF 2014 TACF B3F3	S2-A	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	.	.	17	0.85
14-145	B3F3	JWSF 2014 TACF B3F3	S2-B	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	.	.	18	0.90
14-136	B3F3	JWSF 2014 TACF B3F3	S3-A	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	.	.	17	0.85
14-136	B3F3	JWSF 2014 TACF B3F3	S3-B	0	1	0	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	.	.	16	0.80
14-119	B3F3	JWSF 2014 TACF B3F3	S4-A	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	.	.	18	0.90
14-119	B3F3	JWSF 2014 TACF B3F3	S4-B																							0	
14-131	B3F3	JWSF 2014 TACF B3F3	S5-A	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0	1	1	1	19	0.86
14-131	B3F3	JWSF 2014 TACF B3F3	S5-B	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	21	0.95
14-110	B3F3	JWSF 2014 TACF B3F3	S6-A	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	.	.	18	0.90
14-110	B3F3	JWSF 2014 TACF B3F3	S6-B	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	20	0.91
14-108	B3F3	JWSF 2014 TACF B3F3	S7-A	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	.	.	18	0.90
14-108	B3F3	JWSF 2014 TACF B3F3	S7-B	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	.	.	18	0.90
14-149	B3F3	JWSF 2014 TACF B3F3	S8-A	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1	.	.	17	0.85
14-149	B3F3	JWSF 2014 TACF B3F3	S8-B	1	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1	1	18	0.82

Table 4D. Comparison of TACF "R" and "S" individuals screened with 11 SSR's. Note this data based on *Cd* alleles present rather than *Cm*. Trees were grown at Meadowview, VA - 12 years old on average - and maintained a live stem inoculated for at least 2 to 3 years (see Table 1B for BLUP scores). Susceptible "S" trees were of similar age and had poor BLUP scores and.

TACF ID	Spp. / Genotype	Provider	R / S	LGF_g18 03		LGF_g18 04		LGA_g14 20		LGA_g84 59		LGA_g41 71		LGB_g22 12		LGD_g11 64		LGG_g62 52		LGI_g330 3		LGB_g10 84		LGG_g23 09		Total *Cd* Alleles	*Cd* Allelic Avg
D6-27-20	B3F3 (Clapper)	TACF Meadowview, VA	R1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	22	1.00
D1-27-54	B3F3 (Clapper)	TACF Meadowview, VA	R2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	22	1.00
D3-17-73	B3F3 (Clapper)	TACF Meadowview, VA	R3	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	**0**	**0**	0	1	0	1	10	0.45
D5-20-15	B3F3 (Clapper)	TACF Meadowview, VA	R4	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	18	0.82
D4-9-105	B3F3 (Clapper)	TACF Meadowview, VA	R5	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	11	0.50
D3-18-61	B3F3 (Clapper)	TACF Meadowview, VA	R6	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	20	0.91
D6-27-4	B3F3 (Clapper)	TACF Meadowview, VA	R7	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	.	.	19	0.95
D1-17-99	B3F3 (Clapper)	TACF Meadowview, VA	R8	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	**0**	**0**	0	1	0	1	10	0.45
D5-26-54	B3F3 (Clapper)	TACF Meadowview, VA	S1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	.	.	19	0.00
D2-10-18	B3F3 (Clapper)	TACF Meadowview, VA	S10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	22	1.00
D5-29-124	B3F3 (Clapper)	TACF Meadowview, VA	S2	1	1	0	1	1	1	.	.	0	1	0	1	1	1	0	1	1	1	1	1	1	1	16	0.73
D2-29-122	B3F3 (Clapper)	TACF Meadowview, VA	S3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	21	0.95
D5-1-4	B3F3 (Clapper)	TACF Meadowview, VA	S4	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	21	0.95
D4-26-43	B3F3 (Clapper)	TACF Meadowview, VA	S5	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	21	0.95
D4-11-98	B3F3 (Clapper)	TACF Meadowview, VA	S6	.	.	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	19	0.95
D4-12-29	B3F3 (Clapper)	TACF Meadowview, VA	S7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	21	0.95
D4-29-72	B3F3 (Clapper)	TACF Meadowview, VA	S8	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	19	0.86
D4-17-59	B3F3 (Clapper)	TACF Meadowview, VA	S9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	22	1.00

Table 5. Results of our final test pooling up to 10 trees with 15 SNP markers on the fraction of American (*Cd*) alleles of various susceptible and resistant chestnut trees we have. DNA from the indicated number of trees was extracted pooled together and sequenced. The most resistant tree in the IN-TACF program (JWSF 3A tree) was included as a highly resistant single individual BC3 check and was one of the 10 trees of varying resistance in a pool.

Pools	Genotype(s)	No. Trees Pooled	State Growing Trees	Field Resistance to Blight	American (*Cd*) allele fraction of 15 SNP Markers
American	AC	∞	IN	Highly Susceptible	1.00*
F1	A × C	7	IN	Very Resistant	0.50
Chinese	CC	10	IN	Highly Resistant	0.00
TACF-R-B3F3	B3F3	8	VA	Resistant	0.69
TACF-S-B3F3	B3F3	10	VA	Susceptible	0.93
IN-TACF -R- JWSF - TACF- B3F3	B3F3	10	IN	Resistant	0.90
IN-TACF -S- JWSF - TACF- B3F3	B3F3	10	IN	Susceptible	0.93
IN-TACF -R-JWSF '3A' - B3F1	B3F1	1	IN	Resistant	0.78
IN-TACF -JWSF '3A' & other R B1- B3's	B1F1,B3F1,B3F2,B3F3	10*	IN	Mixed Resistance	0.84**

* Estimated from previous data. Americans have shown no *Cm* resistance alleles when run well; conversely, only susceptible *Cd* alleles at these 15 loci.

** Includes the JWSF '3A' selection which alone scored 0.78 (listed above).

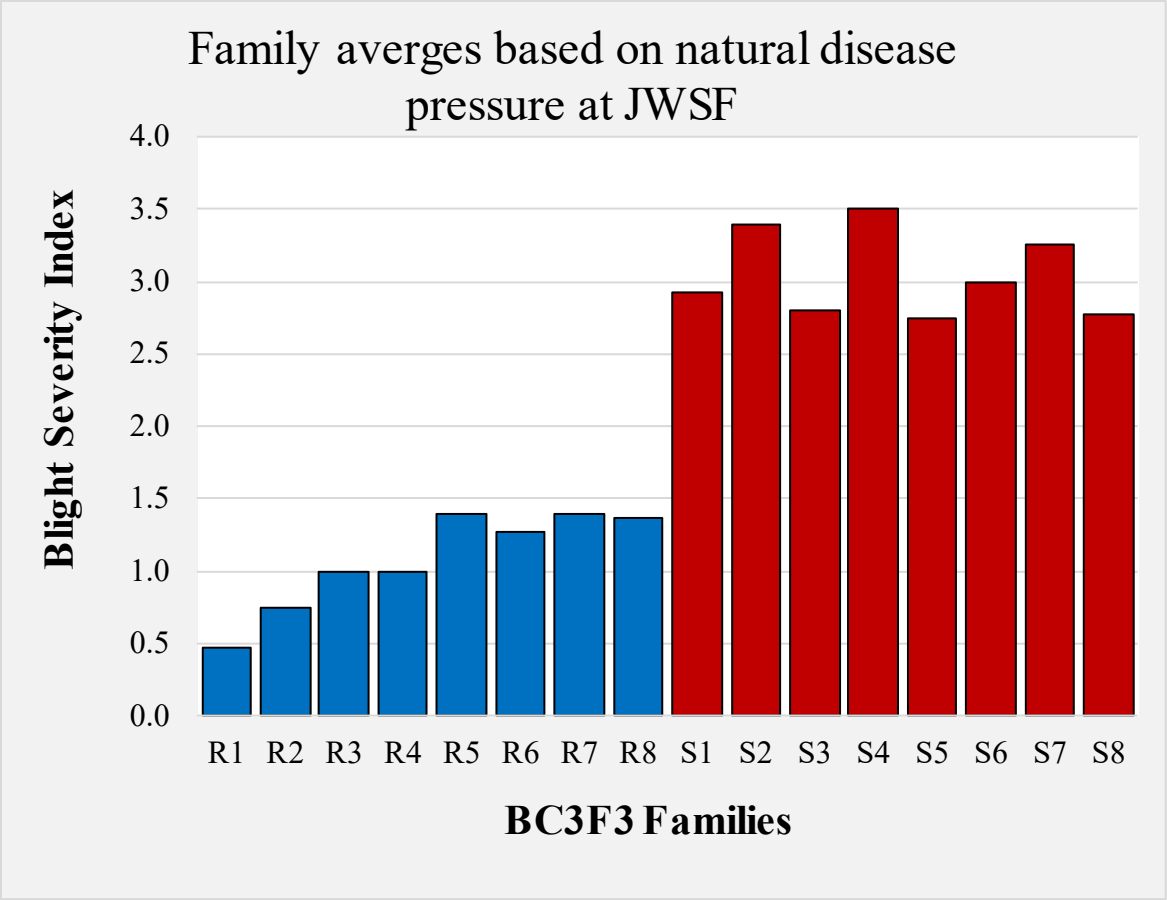

Figure 3. Natural chestnut blight disease severity from 0 (no disease) to 3.0 (every seedling diseased with large cankers) of BC3F3 families ranked as resistant and susceptible selections for screening SNP markers. Blight began in 2015 one year after planting as this JWSF 2014 progeny test is planted next to an 2003 N-TACF BC3F1 orchard that was inoculated in 2008 and has been endemic with chestnut blight since 2012.

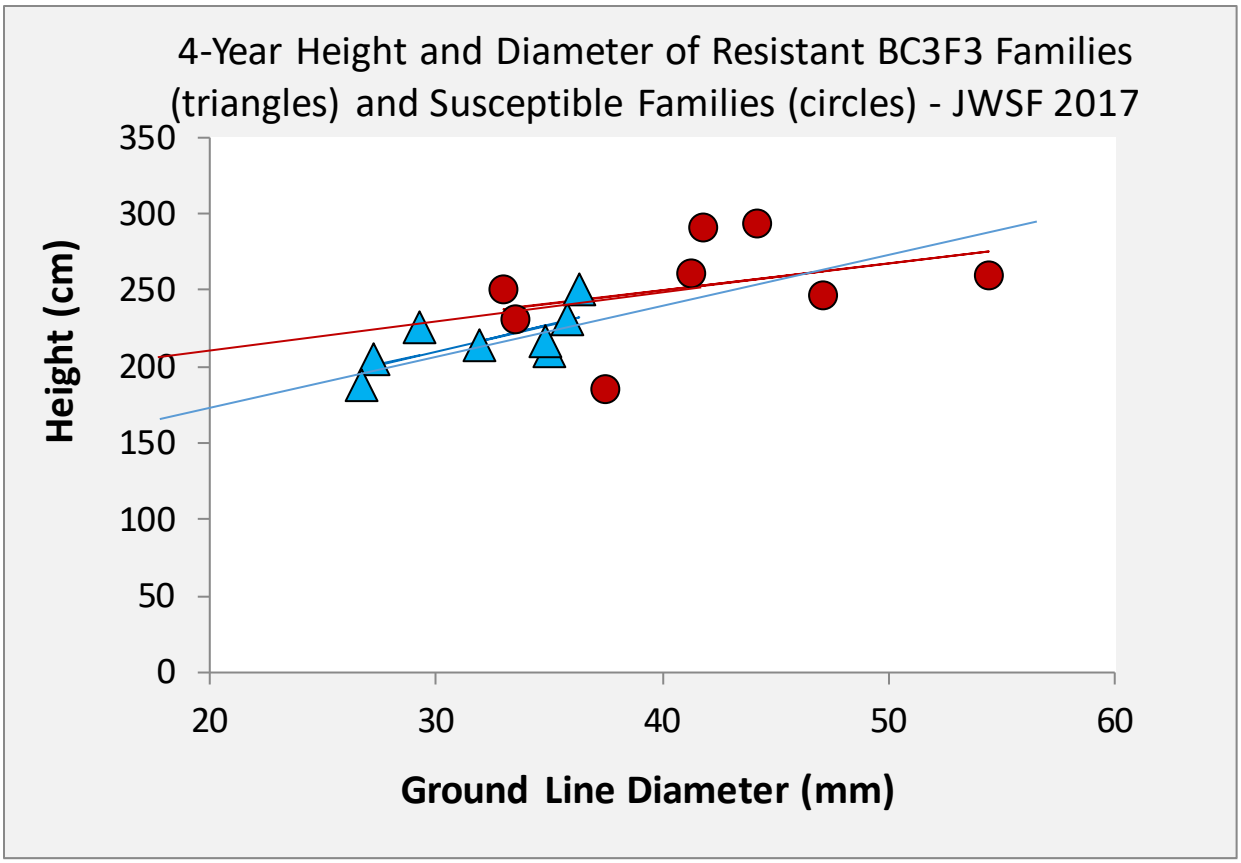

Figure 4. The variation in growth (height and diameter) of BC3F3 families selected as resistant and susceptible selections for screening SNP markers. In general, disease free families tended to be smaller relative to the most susceptible.

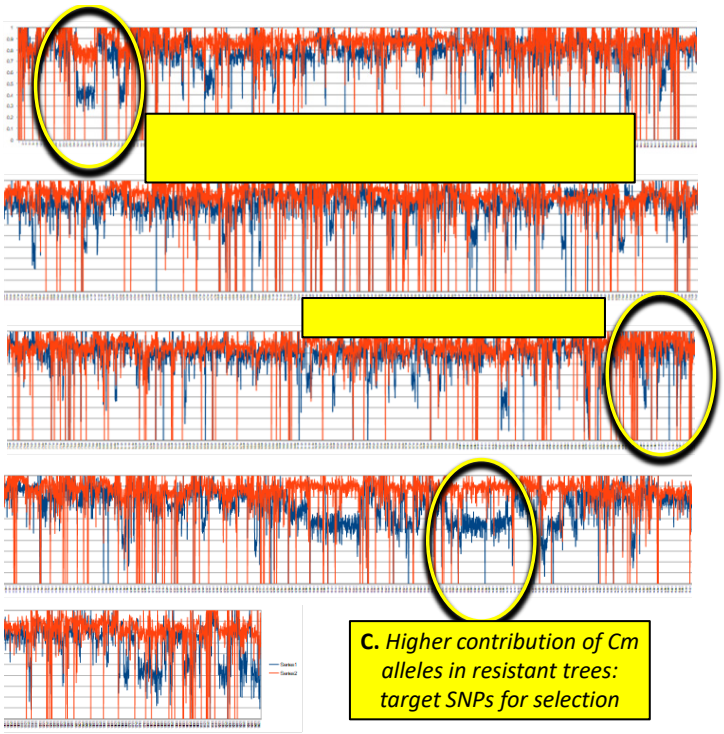

Figure 5. The variation in the frequency of both *Cm* and *Cd* alleles throughout the chestnut genome from all our pooled sequences of all our resistant and susceptible backcrosses for all 15 SNP markers illustrating the complexity introgressing resistance alleles from Chinese to American.

Table 6. 16 American chestnut trees grafted by the HTIRC and IN-TACF in the J.S. Wright Center greenhouse at the Purdue FNR Martell Forest april - May 2018.

HTIRC-TAG ID	Tenn. ID-2	ID-3	Rootstocks	Genotype	R-STATUS	Scion County	Scion State	Scion (ortet) Site	Row-	Tree	No. Grafts	2018 Successful Grafts	2018 % Take
18-21	ALCLEB-04	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Cleburne	AL	Frames Property	-	-	4	2	0.50
18-22	ALCLEB-06	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Cleburne	AL	Frames Property	-	-	5	2	0.40
18-23	ALCLAY-01	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Clay	AL	Sonny Clarke Property	-	-	5	0	0.00
18-24	ALCALH-27	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Calhoun	AL	Choccolocco Mtn.	-	-	5	3	0.60
18-25	ALJEFF-82	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Talladega	AL	Adams Gap	-	-	4	0	0.00
18-26	ALJEFF-83	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Talladega	AL	Adams Gap	-	-	5	1	0.20
18-27	ALJEff-84	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Talladega	AL	Adams Gap	-	-	5	0	0.00
18-28	ALJEFF-74	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Jefferson	AL	Ruffner Mtn.	-	-	5	1	0.20
18-29	ALJEFF-24	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Jefferson	AL	Ruffner Mtn.	-	-	5	0	0.00
18-30	ALJEFF-14	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Jefferson	AL	Ruffner Mtn.	-	-	4	1	0.25
18-31	TNCAN-01	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Cannon	TN	Todd Jr. Property	-	-	5	2	0.40
18-32	TNCAN-02	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Cannon	TN	Todd Jr. Property	-	-	5	1	0.20
18-33	TNHEN-01	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Henderson	TN	Natchez Tract	-	-	5	0	0.00
18-34	TNHEN-02	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Henderson	TN	Natchez Tract	-	-	4	0	0.00
18-35	TNHEN-06	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S	Henderson	TN	Natchez Tract	-	-	5	1	0.20
18-36	LACON-19	-	*C. dentata - IN-TACF Duke Orch Mix*	AC	H-S		AL		-	-	4	1	0.25
											75	**15**	**20%**

Table 7. 14 other American chesntut trees grafted by the HTIRC and IN-TACF in the JL Block of the Duke Orchard at the Purdue FNR Martell Forest June 2018.

HTIRC-TAG ID	Tenn. ID-2	ID-3	Rootstocks	Genotype	R-STATUS	Scion County	Scion State	Field Graft Orch	Row-	Tree	No. Grafts	2018 Successful Grafts	2018 % Take
18-50	ALJEFF-72	-	JL Bark Graft - 4-yr AC sdlg	AC	H-S	Talladega	AL	JL Bark Graft	-	-	2	2	1.00
18-51	ALJEFF-79	-	JL Bark Graft - 4-yr AC sdlg	AC	H-S	Talladega	AL	JL Bark Graft	-	-	1	0	0.00
18-52	ALJEFF-14	-	JL Bark Graft - 4-yr AC sdlg	AC	H-S	Talladega	AL	JL Bark Graft	-	-	0	0	
18-53	ALJEFF-78	-	JL Bark Graft - 4-yr AC sdlg	AC	H-S	Talladega	AL	JL Bark Graft	-	-	1	1	1.00
18-54	ALCALH-02	-	JL Bark Graft - 4-yr AC sdlg	AC	H-S	Calhoun	AL	JL Bark Graft	-	-	1	1	1.00
18-55	ALJEFF-80	-	JL Bark Graft - 4-yr AC sdlg	AC	H-S	Talladega	AL	JL Bark Graft	-	-	2	1	0.50
18-56	ALJEFF-25	-	JL Bark Graft - 4-yr AC sdlg	AC	H-S	Talladega	AL	JL Bark Graft	-	-	2	1	0.50
18-57	LACON-10	-	JL Bark Graft - 4-yr AC sdlg	AC	H-S		AL	JL Bark Graft	-	-	2	1	0.50
18-58	TNHEN-05	-	JL Bark Graft - 4-yr AC sdlg	AC	H-S	Henderson	TN	JL Bark Graft	-	-	2	2	1.00
18-59	TNHEN-03	-	JL Bark Graft - 4-yr AC sdlg	AC	H-S	Henderson	TN	JL Bark Graft	-	-	1	1	1.00
18-60	ALCALH-01	-	JL Bark Graft - 4-yr AC sdlg	AC	H-S	Calhoun	AL	JL Bark Graft	-	-	1	1	1.00
18-61	ALJEFF-76	-	JL Bark Graft - 4-yr AC sdlg	AC	H-S	Talladega	AL	JL Bark Graft	-	-	1	1	1.00
18-62	ALJEFF-81	-	JL Bark Graft - 4-yr AC sdlg	AC	H-S	Talladega	AL	JL Bark Graft	-	-	1	1	1.00
18-63	ALCALH-22	-	JL Bark Graft - 4-yr AC sdlg	AC	H-S	Calhoun	AL	JL Bark Graft	-	-	1	0	0.00
											18	**13**	**72%**

Table 8. Individual BC3F3 selections from the best progeny from the eight least blighted families in the 2014 JWSF Indiana progeny test grafted by the HTIRC and IN-TACF in the J.S. Wright Center greenhouse at the Purdue FNR Martell Forest april - May 2018.

HTIRC-TAG ID	TACF ID-2	ID-3	Rootstocks	Scion Genotype	R-STATUS	County	State	Scion Site/Orch	Row-	Tree	No. Grafts	2018 Successful Grafts	2018 % Take
18-37	D1-17-4	R2-B	/2 BC3F2 (Line 3A) - 1/2 BC1F1 (Line 4A	14-104	R	Washington	IN	14-JWSF-BCF3	9	27	8	4	0.50
18-38	D3-29-1	R1-D	/2 BC3F2 (Line 3A) - 1/2 BC1F1 (Line 4A	14-125	R	Washington	IN	14-JWSF-BCF3	8	9	6	6	1.00
18-39	D4-20-65	R4-B	/2 BC3F2 (Line 3A) - 1/2 BC1F1 (Line 4A	14-129	R	Washington	IN	14-JWSF-BCF3	8	24	6	4	0.67
18-40	D4-20-65	R4-A	/2 BC3F2 (Line 3A) - 1/2 BC1F1 (Line 4A	14-129	R	Washington	IN	14-JWSF-BCF3	3	36	6	5	0.83
18-41	D1-21-25	R5-A	/2 BC3F2 (Line 3A) - 1/2 BC1F1 (Line 4A	14-105	R	Washington	IN	14-JWSF-BCF3	7	36	6	4	0.67
18-42	D6-26-27	R6-A	/2 BC3F2 (Line 3A) - 1/2 BC1F1 (Line 4A	14-142	R	Washington	IN	14-JWSF-BCF3	8	8	6	3	0.50
18-43	D1-28-19	R7-A	/2 BC3F2 (Line 3A) - 1/2 BC1F1 (Line 4A	14-113	R	Washington	IN	14-JWSF-BCF3	2	10	6	4	0.67
18-44	D1-26-105	R8-A	/2 BC3F2 (Line 3A) - 1/2 BC1F1 (Line 4A	14-107	R	Washington	IN	14-JWSF-BCF3	21	36	6	4	0.67
18-45	D5-18-101	1-*	BC1F1 (Line 4A)	14-137	R	Washington	IN	14-JWSF-BCF3	5	11	4	3	0.75
18-46	D5-18-101	2-*	BC1F1 (Line 4A)	14-137	R	Washington	IN	14-JWSF-BCF3	11	36	4	1	0.25
18-47	D5-18-101	3-*	BC1F1 (Line 4A)	14-137	R	Washington	IN	14-JWSF-BCF3	12	43	4	1	0.25
18-48	-	LINE-3A	/2 BC3F2 (Line 3A) - 1/2 BC1F1 (Line 4A	JWSF '03-3A	H-R	Washington	IN	14-JWSF-BCF3	'03	B3F1	8	3	0.38
18-49	-	F2-LINE-2-*M*	2/8 BC3F2 (Line 3A) - 6/8 BC1F1 (Line 4A	R17-T4-F2	R	Washington	IN	14-JWSF-BCF3	9	32	8	7	0.88
** These half-sib BC3 seedlings stood out with outstanding American form*											**78**	**49**	**63%**