NE-1333 Technical Committee Meeting
Biological Improvement of Chestnut through Technologies that Address Management of the Species, its Pathogens and Pests

Cataloochee Ranch, Maggie Valley, NC
September 7-9, 2017

Attendance:

Alabama:	David and Judi Morris (AL Chapter, TACF)
Kentucky:	Albert Abbott (University of Kentucky)
Mississippi:	Angus Dawe, Didi Ren (Mississippi State University)
New York:	Linda McGuigan, Dakota Matthews (SUNY-ESF)
North Carolina:	Paul Sisco, Ben Jarrett, Jarrod Westbrook (TACF®, Asheville)
Pennsylvania:	John Carlson (chair-elect) (Pennsylvania State University)
Portugal:	Rita Costa (INIAV, Oeiras)
South Carolina:	Steve Jeffers, Tatyana Zhebentyayeva (Clemson University)
Tennessee:	Hill Craddock (Chair), Kirsten Hein, Taylor Perkins (UT Chattanooga)
Virginia:	Fred Hebard (TACF®, Meadowview), Laurel Rodgers (Shenandoah University)
West Virginia:	William MacDonald, Mark Double, Cameron Stauder (West Virginia University)

The meeting was called to order by chairman Hill Craddock at 9:00 am on 8 Sept 2017 at the Cataloochee Guest Ranch in Maggie Valley, NC. Craddock introduced Ben Jarrett, the new southeast coordinator the The American Chestnut Foundation. Jarrett is housed in Asheville, NC and he will oversee activities in the Carolinas, Alabama, Georgia and Tennessee.

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<u>OBJECTIVE 1</u>. ***To develop and evaluate blight resistant chestnut trees for food and fiber through traditional and molecular techniques that incorporate knowledge of the chestnut genome***

Jared Westbrook, The American Chestnut Foundation® (Asheville)

Background and rationale. For over 30 years, The American Chestnut Foundation has been selecting for chestnut blight resistance in American chestnut backcross populations by inoculating trees with the fungus that causes chestnut blight (*Cryphonectria parasitica*) and culling all trees except for those with the least severe cankers. Over 60,000 American chestnut backcross trees have been planted in TACF's Meadowview seed orchards since 2002. After inoculation and culling, 5000 trees remain from which to make the final selections of 500 of the most disease resistant trees.

As we continue to select against susceptible trees, it becomes increasingly difficult to distinguish the most resistant trees by sight alone. A tree's apparent disease resistance is a function of the resistance genes that it inherited and the environment in which it is growing. To separate the effect of genes and environment on disease resistance, 30–50 progeny of mother trees in TACF seed orchards are planted in randomized field trials that are known as *progeny tests.*

The genetic levels of blight resistance of these mother trees may be estimated from the average canker severity of their progeny. The effect of environment on disease resistance may be accounted for by replicating progeny in multiple environments. Progeny testing also enables us to screen for resistance to Phytophthora root rot (PRR), a second disease that kills American chestnut. Currently, the orchards at Meadowview are not affected by PRR. Resistance to PRR is screened off site to prevent the disease from being established at Meadowview.

Accelerated progeny test for blight resistance with a small stem assay. A knit-picker is used to wound small stems after which an agar plug of inoculum is secured in place with parafilm. Progeny testing is being conducted to determine how genetically blight-resistant the 10% of BC_3F_2s are in seed orchards. The goal is to get down to the best 1% by screening the progeny of the remaining trees. They plant 30-50 trees in field trials; those are inoculated and screened for blight resistance. Why conduct progeny tests on small stems? Results can be obtained within 1 year in greenhouse tests versus 3 years for field results. Samples can be increased within families beyond what is feasible in the field. The small stem assay requires less labor and cost.

The 2017 small stem assay experiment design was as follows:

- 69 BC_3F_3 families plus Chinese and American chestnut controls were used.
- 40 seeds were sowed per family.
- Randomized block design was used.
- 1/2 of the BC_3F_3s inoculated with SG2-3 (less virulent) and 1/2 inoculated with Ep155 (more virulent).

Approximately 2840 seeds were sowed. After reviewing the results of both *C. parasitica* isolates, it was decided to use only Ep155 and not SG2-3 and take measurements at 15 wks.

Canker length is heritable among the BC_3F_3 families. How does genetic variation in canker length among BC_3F_3 families compare with Chinese and American chestnut? There are

large error bars, but there is a difference between American and Chinses chestnut and it is possible to distinguish the highly resistant BC_3F_3 families.

Is BC_3F_3 family genetic variation in canker length correlated between the small stem assays and orchard progeny tests? There was a positive genetic correlation (0.84 ± 0.74) between canker lengths of 13 BC_3F_3families inoculated with *C. parasitica* in orchard and small stem assays. Error bars in the correlation was high presumably because few families have yet to be evaluated in both orchards and small small stem assays. In trying to optimize the small stem assay for progeny testing, Westbrook is concerned about the number of "no take" inoculations in 2017.

Even with accelerated progeny testing using the small stem assay, progeny testing alone is too slow to finish the selection for blight resistance in seed orchards. There are over 5000 trees remaining in Meadowview seed orchards; only 600 have been progeny tested since 2011. The rate of progeny testing is limited by the rate at which trees in seed orchards reach reproductive maturity and begin producing seeds. Since the planting of seed orchards started in 2002, approximately 1000 trees of tens of thousands of trees planted have flowered and produced seed for progeny testing.

Fortunately, there is another way to make accurate selection of the most genetically resistant trees - *genomic selection*. The basic principle of genomic selection is to develop a prediction model that enables us to rank trees' disease resistance with DNA sequencing. 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 underlying genetic resistance of mother trees that have not been progeny tested 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. The principle advantage of genomic selection is that it will enable us to accelerate selection of the most disease resistant trees.

As a proof-of-concept for genomic selection, genotyping-by-sequencing has been performed on 480 Graves mother trees from Meadowview seed orchards. Over 20,000 SNPs were detected in this population. Genomic prediction models for blight resistance were first developed by estimating the relationships between the SNPs and variation in subjective canker severity rating of these individual trees. The predictive ability of the SNPs was estimated with cross-validation. The effects of SNPs on canker severity was first estimated in $9/10^{ths}$ of the population. The SNPs effects were then multiplied by the number of SNP alleles in remaining $1/10^{th}$ of the population and summed to obtain genomic predictions of canker severity. This procedure was repeated nine more times with different partitions of the population into training and prediction sets to obtain canker severity predictions for all individuals.

The strength of the correlation between predicted and observed canker severity were used to estimate genomic predictive ability. The predictive ability for raw canker ratings was only 0.2 on a 0 to 1 scale. Canker ratings are influenced by genetic and environmental effects, including the bias of the people rating the cankers. To more accurately estimate the underlying genetic resistance, the effects of seed orchard block and year of inoculation were accounted for. Then, the canker ratings of siblings and progeny were factored in a weighted average of canker ratings for each individual tree to estimate the underlying resistance. The predictive

ability of the SNPs increased to 0.6, presumably because genetic resistance was more accurately estimated in the training population.

The end goal is to use progeny test data to develop genomic prediction models. As a proof-of-concept, genomic prediction was performed with 47 BC_3F_2 mother trees whose underlying genetic resistance to chestnut blight was estimated from progeny tests. Between 11 and 30 progeny of each of these mother trees were inoculated with the chestnut blight fungus. Genetic variation in resistance of the mother trees was estimated from the average canker length and canker ratings of the progeny. Genomic predictive abilities were near zero for the average canker ratings of the progeny but approximately 0.55 and 0.4 for canker lengths from inoculations with weak and strong strains of the chestnut blight, respectively. Genetic variation in PRR was also predicted with genomics. Predictive abilities were higher for the severity of root rot lesions and mortality (0.65) than for above ground wilting (0.45).

These results suggest that traits differ in how accurate they represent underlying genetic levels of disease resistance – canker length more accurately represents blight resistance than canker rating. Mortality and severity of root lesions represents PRR resistance more accurately than above ground wilting. The genomic predictive abilities for chestnut blight canker length, root rot severity, and root rot mortality were encouragingly high despite the small training populations used to develop the prediction models.

Next steps. Genomic predictive abilities are expected to increase by genotyping more progeny tested trees. TACF is collaborating with Professor Jason Holliday at Virginia Tech to genotype all mother trees in Meadowview seed orchards that have been progeny tested for resistance to chestnut blight and/or PRR. These trees will be the training populations to develop genomic prediction models for resistance to these pathogens. Approximately 300 Clapper and 300 Graves mother trees will have been progeny tested for blight resistance by 2019. In addition, 300 Graves mother trees have been progeny tested for PRR resistance. Separate genomic prediction models will be developed for Graves and Clapper because of the possibility that these sources of resistance inherited different disease resistance genes.

In addition, 1200 Clapper and 800 Graves trees from Meadowview seed orchards that remain after the initial culling of the most blight-susceptible trees, but that have not yet been progeny tested, also are being genotyped. Once all of the individuals in the population are ranked with respect to blight or PRR resistance, final selections will be made of individuals with the highest disease resistance. The rest of the individuals will be culled from the orchard.

To test accuracy of genomic prediction models developed at Meadowview for selection in TACF's chapter seed orchards, we are also progeny testing and genotyping BC_3F_2 mother trees from chapter seed orchards. Predictions of resistance in chapter seed orchards will be made from Meadowview prediction models. The accuracy of those predictions will be estimated from the correlation with progeny test rankings of disease resistance.

Tatyana Zhebentyayeva, Clemson University

An update on QTL mapping of resistance to *P. cinnamomi* in biparental American X Chinese chestnut crosses. *Phytophthora cinnamomi* is a lethal, soil-borne pathogen of many plant species, including American chestnut. Asian *Castanea* species are resistant. Because *P. cinnamomi* is found in many locations in the southeastern US as far north as Pennsylvania, chestnut restoration efforts of The American Chestnut Foundation® include breeding resistance

to this pathogen as well as to chestnut blight. Knowledge of the genetics of resistance to *P. cinnamomi* in Asian species will aid in development of an efficient and successful breeding program.

Previously, genome-wide QTL analysis of a small 'Nanking' BC1 family revealed two non-overlapping QTLs on Linkage Group E (Table 1, Line 1). Subsequently Zhebentyayeva, using SSRs known to be on Linkage Group E, found more evidence for two QTLs on Linkage Group E in other BC_1 families derived from both 'Mahogany' and 'Nanking' (Table 1, Lines 2-5, Figure 2).

Table 1. Hybrid families used in previous analyses for QTLs associated with resistance to *P. cinnamomi*

Hybrid Family Code - Year	Total Plants	Root rot symptom severity				Type of family	Source of resistance
		0	1	2	3		
KYADA1 x GL158	48	0	0	27	21	BC_1	*C. mollissima* cv. 'Nanking'
HB2 - 2011	41	1	3	20	17	BC_1	*C. mollissima* cv. 'Mahogany'
HB2 - 2012	179	0	2	122	55	BC_1	*C. mollissima* cv. 'Mahogany'
NK1 - 2012	20	1	13	6	0	BC_1	*C. mollissima* cv. 'Nanking'
NK2 - 2012	83	2	32	42	7	BC_1	*C. mollissima* cv. 'Nanking'

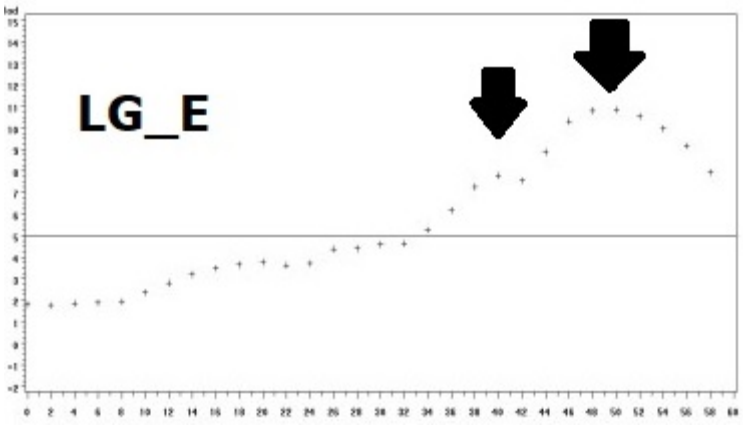

Figure 1. Cumulative LOD plot of QTLs on LG_E determined by SSR analysis (**LG_E only**) of families HB1, HB2, NK1, and NK2

To better elucidate the genetics of resistance to *P. cinnamomi*, Genotype-by-Sequencing (GBS) was used to do a genome-wide analysis of larger hybrid BC_1 and BC_3 families segregating for resistance to *Phytophthora cinnamomi*. The backcross families were derived from Chinese chestnut cultivars 'Mahogany' and 'Nanking', sources of resistance to chestnut blight being utilized by The American Chestnut Foundation®.

Materials and Methods. The BC_1 and BC_3 families were generated by controlled pollination at the Meadowview Research Farms ('Mahogany' BC_1 family HB2), at the Cliffs of Glassy, Landrum, SC ('Nanking' BC_1 family NK4) and in the Cranberry community of Avery

County, NC ('Mahogany' BC_3 family JB1). In April 2013, seed of JB1 were planted in a single tub at the home of Clint Neel in Pegram, TN, and in April 2014, the 2014 families were planted in a replicated, randomized design at the Chestnut Return Farms (Seneca, SC). In July, seedlings were labeled and leaf tissue was harvested and stored in a -80°C freezer at Clemson University. Seedlings were then inoculated with one isolate of *P. cinnamomi* (in Tennessee, Tennessee isolate) and two isolates of *P. cinnamomi* (in South Carolina, South Carolina isolates) and left exposed to the pathogen for the remainder of the growing season. In December or January, when the plants were dormant, resistance to *P. cinnamomi* was scored using a 0 (no lesions) to 3 (dead) scale developed by S.N. Jeffers and J.B. James, based on visual examination of the seedling roots (Table 2).

Table 2. Hybrid families analyzed with Genotype-by-Sequencing in this study

Hybrid Family Code - Year	Total Plants	Root rot symptom severity				Type of family	Source of resistance	
		0	1	2	3			Where screened
HB2 - 2014	237	0	3	106	128	BC_1	*C. mollissima* cv. 'Mahogany'	Seneca, SC
NK4 - 2014	318	2	17	135	164	BC_1	*C. mollissima* cv. 'Nanking'	Seneca, SC
JB1 - 2013	115	2	20	37	56	BC_3	*C. mollissima* cv. 'Mahogany'	Pegram, TN
JB1 - 2014	214	1	4	62	147	BC_3	*C. mollissima* cv. 'Mahogany'	Seneca, SC

At the Clemson University Genomics Institute, DNA was isolated from the leaf tissue, two restriction enzymes were used to generate fragments of appropriate length for sequencing (~200-700 bp), and linkers were added so that each sequence could be referenced to its seedling source. The DNA fragments were then sequenced at the Medical University of South Carolina (Charleston, SC). A large number of Single Nucleotide Polymorphisms (SNPs) were found to distinguish the parental genotypes of the BC_1 families, as many as 84,000 SNPs for the 'Nanking' NK4 family. A subset of SNPs was chosen, based on the amount of missing data in the seedlings composing each family. The final group of SNPs had less than 10% missing data in any one seedling. Genetic maps were generated using JoinMap4.1 (van Ooijen, 2006) and QTLs were identified using MapQTL6.0 (van Ooijen, 2004).

Results. Four linkage groups corresponding to four of the 12 chromosome pairs of chestnut were found to have significant QTLs for resistance to *P. cinnamomi*. The non-parametric Kruskal–Wallis (KW) test was employed to detect association between markers and traits individually. In a second step, interval mapping (IM) analysis was performed to select markers significantly associated with the trait to find an initial set of cofactors. A backward elimination procedure was applied to the initial set of cofactors. Using a function of MapQTL6.0, the most significant markers were selected and used as cofactors in a multiple QTL method (MQM) analysis for QTL detection. A mapping step size of 1 cM was used for both the IM and MQM analyses. The LOD (Log of odds) thresholds for genome-wide QTL detection were empirically determined based on the Permutation Test with 1,000 iterations. A threshold LOD

value of 2.8 was used to declare the presence of a QTL. Regions with a LOD score above 2.0 were also inspected for potential QTLs if in one of the two crosses significant signal was detected nearby.

Table 3. Detailed genetics maps for both the male and female parents of HB2, NK4, and JB1 families, allowing the ordering of >3000 scaffolds in the *C. mollissima* reference map.

Genetic Map	Number of Scaffolds	Total Mb	Resistance Donor
HB2-2014	921	57.7	'Mahogany'
NK4-2014	1600	89.6	'Nanking'
JB1-2014	1904	111.7	'Mahogany'
Total	3186	174.5	

Discussion. The results of this study clearly showed: (1) that more than one locus from *C. mollissima* was correlated with resistance to *P. cinnamomi* in these hybrid families; and, (2) different subsets of loci were correlated with resistance in each cultivar. In the HB2 family derived from 'Mahogany', loci on LGs A, E, and K were significant in the MQM mapping, whereas in the NK4 family derived from 'Nanking, loci on LGs C and E were significant, with a locus on LG K identified as just below the significance level. In the JB1 BC_3 family, only the two previously-identified QTLs on LG_E were significant.

LG_E appeared to have more than one significant locus, confirming previous work by Tom Kubisiak and Bode Olukolu (Kubisiak, 2010; Olukolu et *al*. 2012). The most significant locus in the HB2 family was near the central part of LG_E, whereas the most significant locus in the NK4 and JB1 families was near the distal end of one arm of LG_E. The NK4 family also had a less significant locus near the central part of LG_E, perhaps the same locus as the significant one in the HB2 family.

Future work will focus on narrowing down the significant loci identified in this study with the goal of finding useful molecular markers for screening for resistance to *P. cinnamomi* in seedlings. A 'Nanking' F_2 family of 325 seeds has also been phenotypically screened for resistance to this pathogen, which will help to identify any recessive factors in disease resistance.

Mark Double, West Virginia University

BC_3F_3 Planting at the University Forest, Morgantown, WV. Two hundred advanced backcross seedlings (BC_3F_3) were planted in April/Sept 2015 at the University Forest near Coopers Rock in Preston County. WVU forestry students, members of the Urban Forestry Club, helped with the planting. An additional 100 backcross seedlings were planted in October 2016. Tress were assessed in Aug 2017 with the results listed in the following table.

Lot	# Dead/Total	% Dead	Planted	Type
1=D8-10-19	4 of 14	29%	11-Apr-15	Seed
2=D2-29-55	4 of 14	29%	11-Apr-15	Seed
3=D3-8-119	2 of 12	17%	11-Apr-15	Seed
4=D7-26-86	5 of 12	42%	11-Apr-15	Seed
5=D5-17-89	5 of 11	45%	11-Apr-15	Seed

6=D6-29-148	2 of 8	25%	9-Jun-15	Potted seedlings
7=D4-11-52	0 of 9	0%	9-Jun-15	Potted seedlings
8=W2-30-124	2 of 7	29%	9-Jun-15	Potted seedlings
9=D3-29-14	2 of 6	33%	9-Jun-15	Potted seedlings
10=W15-150	2 of 7	29%	9-Jun-15	Potted seedlings
11=W2-32-108	19 of 32	59%	Sep-16	Potted seedlings
12=W5-21-9	12 of 29	41%	Sep-16	Potted seedlings
13=W5-31-108	9 of 22	41%	Sep-16	Potted seedlings
14=D4-27-78	3 of 17	18%	Sep-16	Potted seedlings
Lot C	38/100	38%	5-Dec-15	Bareroot seedlings

Backcross orchard for assessment of host resistance combined with hypovirulence. W.L. MacDonald and M.L. Double (in cooperation with Fred Hebard and Sara Fitzsimmons, The American Chestnut Foundation)

Six replicate plots each containing 150 trees have been established at the Plant and Soil Sciences Farm in Morgantown, WV to assess the interaction of host resistance and virulent/ hypovirulent strains of *Cryphonectria parasitica*. To establish the planting, seeds were planted annually from 2006-2011. In three plots, naturally occurring cankers were treated with hypovirulent isolates; three plots were not inoculated. As of July 2015, overall survival was 70%; that dropped to 68% in 2017. Living trees in the non-treated and hypovirulent-treated plots were rated in July 2017. Data are listed in the following table.

	Non-Treated Plots		**Treated Plots**	
Species/BC	**Total**	**Alive**	**Total**	**Alive**
American	92	55%	90	68%
BC_2F_2	54	54%	57	93%
BC_2F_3	63	60%	66	70%
BC_3F_2	72	50%	62	68%
Chinese	97	92%	92	91%
European	72	25%	83	58%

All naturally-occurring cankers in the three hypovirus-introduction plots were treated during the 2013-2017 growing seasons with a hypovirulent slurry (Euro 7, COLI, GH2 and Weekly/Ep155/pXHE7). In 2017, an additional hypovirulent isolate (SR 136-3 Hv) was added so that all *vic* genes were included in the hypovirulent slurry, as shown below.

Isolate	***vic* Genotype**
Weekly Hv	2211-11
COLI	1122-11
GH2	1211-11
Euro 7	2111-11
SR 136-3 Hv	2212-22

The benefit of hypovirus canker treatment to maintain trees is clearly demonstrated, especially for the BC_2F_2 hybrids.

John Carlson, Schatz Center for Tree Molecular Genetics, Pennsylvania State University

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Methods / keywords:

- Genome sequencing; DNA markers; Gene mapping and functional characterization
- Seed orchards; Field trials; Restoration.

<u>Chestnut Genome Sequencing Project Activities</u>

John Carlson, PI, Schatz Center for Tree Molecular Genetics at Penn State
Project team: Nathaniel Cannon and Nicole Zembower at Penn State University; Tatyana Zhebentyayeva at Clemson University; Margaret Staton and Nathan Henry at the University of Tennessee; Bert Abbott and Dana Nelson at the University of Kentucky at Lexington; Jason Holliday and Mihir Mandal at Virginia Tech University; Nurul Islam-Faridi at Texas A&M University; Jared Westbrook and Sara Fitzsimmons at The American Chestnut Foundation.

Project Objective addressed:
Objective 1 "Develop and evaluate blight resistant chestnuts for food and fiber through traditional and molecular approaches that incorporate knowledge of the chestnut genome."

UPDATE:

Version 1.1 of the Chinese chestnut genome is still available to the public at the website https://hardwoodgenomics.org/chinese-chestnut-genome, created and curated by Dr. Margaret Staton at the University of Tennessee-Knoxville. The version 1.1 genome assembly (for TACF cv. Vanuxem) consists of 724.4 Mb in 41,270 scaffolds, averaging app. 40,000 bp in length. A total of 36,146 gene models and 38,146 peptide sequences were predicted in the genome. BAC contigs spanning the 3 blight resistance QTL (identified in the early F2 QTL mapping population) were also sequenced and assembled into 395 scaffolds. A total of 1,952 genes were predicted within the 3 QTLs, including 194 known stress-response genes, from which 15 candidate genes for blight resistance were selected for further study, based on gene expression data.

The anticipated public release of the improved and validated version 2 of the Chinese chestnut genome, is pending journal publication. This assembly consists of 14,358 scaffolds representing 784Mb of genome sequence, almost the estimated genome size. The integrated genetic and physical map was used to distill the 14,358 scaffolds into 12 "pseudo"-chromosome sequences, representing the 12 linkage groups and providing 98% genome coverage. The arrangement of scaffolds in the pseudo-chromosome sequences has been validated by comparison to the order of thousands of DNA markers on new high density genetic linkage maps produced by Tatyana Zhebentyayeva at Clemson, and by comparison to very long PACBio technology genome sequences, with support from a USDA grant awarded to TACF. Jason Holiday's lab at VA Tech University produced RNA sequence data from 9 tissues from grafted clones of the Vanuxem reference genotype, which we have mapped to the new pseudo-chromosomes. All but a few of computer-predicted gene models were validated.

The Chinese chestnut genome was used as a reference to genotype, and assess genetic variation, at the DNA level among genotypes from CAES and TACF orchards. App. 10X depth sequence data was produced in 2015 for: one C. alnifolia genotype, one C. crenata genotype, five C. dentata genotypes (GMBig, Ted Farm A, Alex R, Huan Row1Tree18(MK5), and Ellis 1), one C. henryii genotype (Chinese chinkapin), four C. mollissima genotypes (Mahogany, Nanking, PA Fat Camp, and PA Stone Valley), one C. ozarkensis genotype, one C. sativa genotype, one C. seguinii genotype, three third backcross hybrids from the TACF breeding program (from parents B3119 x B3176), and the BC3 C. dentata x C. mollissima parental genotypes - B3119 and B3176. Alignment of the parental and BC3 genotype genome sequences to the Vanuxem reference genome revealed varying extents of transition of the BC3 genomes towards the American genome content from backcrossing. A similar, but more extensive study of genome sequence variation within and among chestnut species has been conducted in the lab Keith Woeste in the HTIRC at Purdue University.

Jason Holiday and Jared Westbrook are developing a Genome-Wide-Selection model for use in accelerating the TACF back-cross breeding program, with funding from USDA. The Staton group at The University of Tennessee produced a set of 714,039 SNPs from genome sequence data for three American genotypes, which will be used in developing the Genome-Wide-Selection model(s).

PLANS FOR THE COMING YEAR:

Work in the coming year will focus on:

1) The Chinese chestnut pseudochromosome sequences will be released to the public for browsing and downloading at the Hardwood Genomics website - https://hardwoodgenomics.org/chinese-chestnut-genome
2) The Chinese chestnut pseudochromosome sequences will also be 'delivered' immediately to TACF and other collaborators directly or through the Hardwood Genomics website
3) We will submit a refereed journal article on Chinese chestnut reference genome to a high visibility, highly respected research journal such as Nature' (in preparation).

Linda McGuigan, SUNY-ESF

Ecological comparisons of transgenic chestnuts. SUNY-ESF is going through the regulatory process with the FDA, EPA and USDA so their transgenic tree lines can be released. As part of the regulatory process, they are conducting many environmental impact studies including the following areas:

- Terrestrial and aquatic insect feeding
- Leaf litter decomposition and seed germination
- Mycorrhizal colonization
- Metabolomics
- Enzyme activities
- Bumble bee feeding on pollen

Tadpole development. Andy Newhouse looked at wood frog tadpoles this summer since chestnut leaves accumulate in vernal pools. Newhouse used leaves of different species—sugar maple, beech, Chinese chestnut, hybrid chestnuts, non-transgenic chestnut and their 'Darling 4', a high expressing transgenic line. Leaves and tadpoles were added to jars and the jars were replicated. Newhouse had more than 100 jars in the experiment along with controls—no leaves (but alfalfa-based pellets). The study took 6-7 weeks before leg development began. Newhouse found that when there was supplemental food, the tadpoles all developed at the same rate for all leaf types. When the alfalfa-based pellets were removed (just leaves), the tadpoles developed fastest on American chestnut leaves, both transgenic and non-transgenic.

Interaction of chestnut leaves with native plant seeds. Chestnut leaves were added to a mix to loosely simulate decay in order to determine if plant seeds would germinate. Plants seeds included: *Acer, Chicorium* (sunflower family), *Pinus, Gaulthecia* (teaberry; Ericaceae family) and *Elymus* (grass family). There was no difference seen in germination when transgenic American chestnut leaves were applied.

Production issues with tissue culture. In anticipation of going through the regulatory process, SUNY-ESF hired Allison Oakes to grow trees from tissue culture. She has developed an *ex vitro* rooting procedure; however, when the small plantlets are outplanted, the leaves turn yellow and grow very little. The base of the shoot develops a callus ball, and the roots do not grow well. When progeny are grown from nuts, the T1 trees do well, but the results are poor when grown from tissue culture. In order to see if sun was an issue when outplanting, shade houses were established for the transgenic seedlings. The results were similar—the seedlings looked poor. With the help of graduate student Tyler Desmarais, SUNY-ESF is going to build a better shade house next summer with a pot-in-pot system so see if that helps.

Dakota Matthews, SUNY-ESF

Oxalate oxidase—quantification in American chestnut—various methods and results. SUNY-ESF's transgenic American chestnuts have been transformed with the oxalate oxidase (OxO) gene. The gene product converts oxalic acid (*C. parasitica*'s main virulence agent) into hydrogen peroxide and carbon dioxide. Matthews is attempting to quantify oxalic oxidase, as this information is needed for the regulatory process (FDA, USDA, EPA). Matthews is using a colorimetric assay that measures hydrogen peroxide. Matthews is quantifying OxO in chestnut stem, leaf and nut tissue. Transgenic OxO concentrations will be compared to OxO levels in wheat products and germinated wheat seed. There is a limitation of using a colorimetric method in that it is not directly measuring OxO, just a byproduct. In trying to find a better method of quantification, Matthews is using a JEOL cryo, field emission transmission electron microscope that can resolve down to two angstroms. Using this piece of equipment will allow him to immunolabel OxO directly on the plant cell wall in various tissues. He first washed tissue with primary antibodies and following a series of rinses, the second wash was with 3nm gold conjugate. After fixation of the gold conjugate to the antigen and OxO enzyme TEM images are acquired. Using stereological techniques, OxO can be quantified and compared on a per cell basis. Matthews is examining transgenic stem tissue and pollen along with clonal and natural wheat controls.

Bumble bee feeding study. Matthews is identifying chestnut pollen clusters collected from honeybee hives. After chestnut pollen clusters have been identified an approximate ratio of chestnut pollen to other wild pollen will be assigned per collection date. Some of these pollen collections will then be treated with OxO and be fed to bumblebee micro-colonies to test if there are any off target effects on pollinators that might come into contact with future transgenic pollen.

OBJECTIVE 2. To evaluate biological approaches for controlling chestnut blight from the ecological to the molecular level by utilizing knowledge of the fungal and hypovirus genomes to investigate the mechanisms that regulate virulence and hypovirulence in C. parasitica

Angus Dawe, Mississippi State University

Current personnel:
Graduate students – Didi Ren, Soum Kundu
Research Associate – Gisele Andrade

Developing a re-annotated genome sequence to facilitate transcriptomics analyses sand gene identification. Next generation sequencing (NGS) technologies create great opportunities for various genomics, transcriptomics and proteomics projects of non-model organisms. A well-annotated genome reference is critical for these projects like exploring target genes' functions or characterizing gene expression profiles. A practical bioinformatics workflow was designed to perform a new genome annotation and develop a comparison between it and a preexisting annotation. The genome and RNA-seq data of the filamentous fungal plant pathogen *Cryphonectria parasitica* were used to test the workflow, which included experimentally validating the improved quality of the new annotation.

In our MP-FIND workflow (Figure 1), the first step is transcriptome assembly, performed with several well-known tools on raw RNA-seq data. The second step is genome annotation, performed with the MAKER2 pipeline with three Hidden Markov Model (HMM)-based gene predictors (GeneMark-ES, SNAP, Augustus). The last step is the comparison of new and prior annotations, for which we have developed a python script to divide all genes from a preexisting annotation into four categories of comparison with the new data (Match, Similar, Different, Nonexistent) by comparing their coding region (start/end coordinates) and internal domains (InterPro ID) to the new annotation. When testing the preexisting annotation of *Cryphonectria parasitica*, we found that there were 11170 genes annotated in the new annotation compared to 11609 in the previous version. In the Match category, 3800 genes (33%) of the preexisting annotation were perfectly matched with new one; in the Similar category, 4100 genes (35%) were sharing at least one exact CDs and all of the same domains; in the Different category, 3583 genes (31%) were sharing no exact CDs and at least one different domain; in the Nonexistent category, 126 of them (1%) did not exist in the new annotation (Figure 2). From the last three groups, 22 out of 27 (81.5%) manually chosen genes were experimentally validated to support the new annotation using PCR. This has now provided more accurate gene prediction information for *C. parasitica*, which can now be used to better inform molecular approaches to understanding fungal pathogenesis on chestnut.

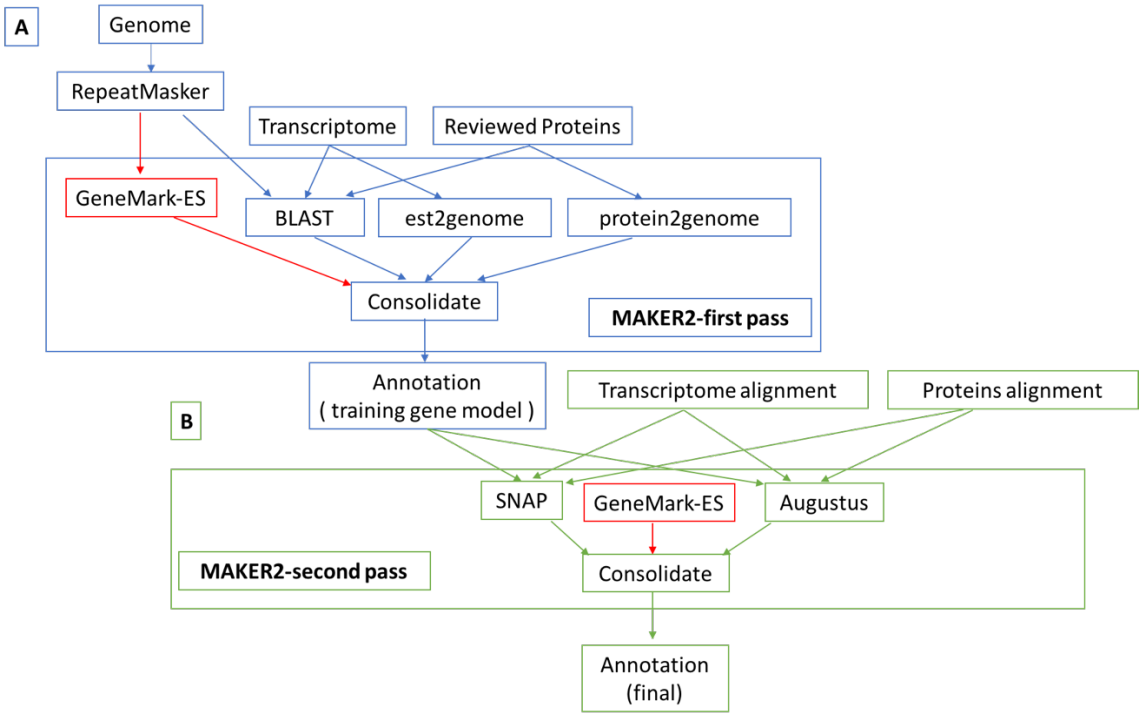

Figure 1 Two pass MAKRE2 genome annotation strategy. (A) In the MAKER2-first pass, an external gene predictor GeneMark-ES were used to provide a training HMM file along with the RNA and proteins evidence for the second pass gene predictors, showing a significant improvement in the number of predicted genes. **(B)** In the MAKER2-second pass, the gene model generated above were applied to train the other gene predictors, SNAP and Augustus along with MAKER2 internal programs to improve the gene model.

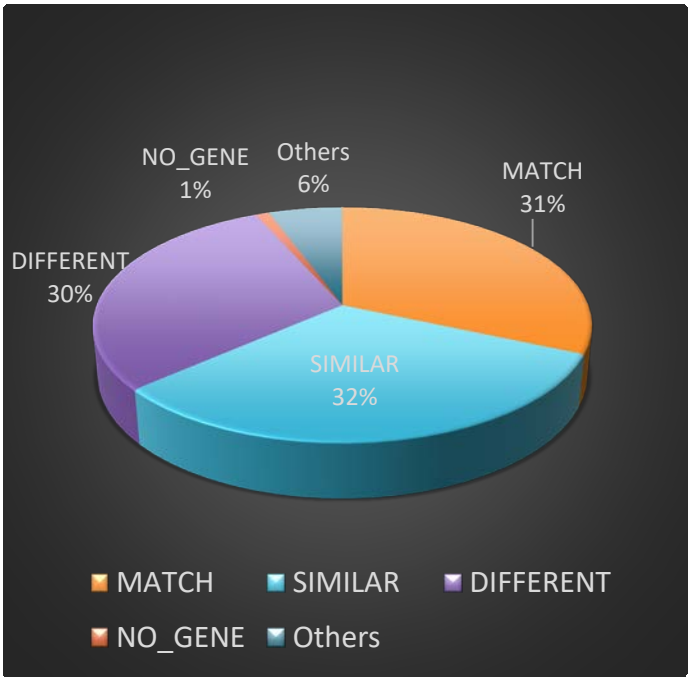

Figure 2. Summary of distributions of the 2009 annotations when compared with 2017. MATCH = 3800 genes (31%). SIMILAR = 3854 genes (32%). DIFFERENT = 3581 genes (30%). NO_GENE = 126 genes (1%). Others = all genes were not predicted in 2017-version but still had some of mRNA alignment support.

Category	Predicted Gene	Annotation version	Gene ID	AED	Coordinates			Predicted Gene size(bp)	Transcript PCR product size(bp)	Positive in PCR	PCR supportive version
					Scaffold	Start	End				
SIMILAR	1	2009	258862	0.48	scaffold_5	920289	920820	531	400	yes	2017 vic 1a-2
		2017	Ep155_U_T00006215_1	0.25	scaffold_5	918882	921612	2730	2261	yes	
	2	2009	356517	1	scaffold_5	924032	924674	642	330	no	2017 vic1b-2
		2017	Ep155_U_T00006216_1	1	scaffold_5	924078	924454	376	292	yes	
	3	2009	255909	1	scaffold_4	1076299	1077428	1129	998	yes	2009 vic4
		2017	Ep155_U_T00004868_1	0.1	scaffold_4	1076177	1077593	1416	1051	no	
	4	2009	231803	0.45	scaffold_3	4888579	4889682	1103	/	/	2017 vic 6
		2017	Ep155_U_T00004505_1	0.11	scaffold_3	4885525	4890764	5239	2000	yes	
	5	2009	355955	0.22	scaffold_4	1069744	1070666	922	/	/	2017
		2017	Ep155_U_T00004864_1	0	scaffold_4	1069009	1070853	1844	817	yes	
	6	2009	263897	0.36	scaffold_7	1689695	1691232	1537	/	/	2017
		2017	Ep155_U_T00008600_1	0	scaffold_7	1688290	1692388	4098	153	yes	
	7	2009	58876	0.4	scaffold_1	678670	679423	753	/	/	2017
		2017	Ep155_U_T00000186_1	0.01	scaffold_1	678481	679500	1019	380	yes	
DIFFERENT	8	2009	98319	0.41	scaffold_5	3000738	3002855	2117	1068	yes	2009, 2017 vic3a-1
		2017	Ep155_U_T00006769_1	0.06	scaffold_5	2993355	3001226	7871	1609	yes	
	9	2009	231853	0.45	scaffold_6	2410893	2412035	1142	902	yes	2009 vic7
		2017	Ep155_U_T00007748_1	0.25	scaffold_6	2408261	2412803	4542	/	/	
	10	2009	102253	0.84	scaffold_5	4277195	4277888	693	1491	yes	2017
		2017	Ep155_U_T00007119_1	0.36	scaffold_5	4274791	4277389	2598	2303	yes	
	11	2009	357202	0.38	scaffold_6	2896210	2897553	1343	/	/	2017
		2017	Ep155_U_T00007829_1	0.05	scaffold_6	2895033	2897647	2614	320	yes	
	12	2009	261603	0.41	scaffold_6	2523249	2525371	2122	570	yes	2009
		2017	Ep155_U_T00007774_1	0	scaffold_6	2522966	2525960	2994	280	yes	
	13	2009	346358	0.36	scaffold_4	4656868	4660251	3383	550	no	2017
		2017	Ep155_U_T00005807_1	0.02	scaffold_4	4657590	4661908	4318	574	yes	
	14	2009	245160	0.38	scaffold_1	3176047	3177203	1156	/	/	2017
		2017	Ep155_U_T00000872_1	0.22	scaffold_1	3172638	3176405	3767	258	yes	
	15	2009	357444	0.43	scaffold_7	1259884	1260550	666	/	/	2017
		2017	Ep155_U_T00008473_1	0	scaffold_7	1258718	1261375	2657	1022	yes	
	16	2009	222652	0.45	scaffold_6	1371443	1372342	899	208	no	2017
		2017	Ep155_U_T00007540_1	0	scaffold_6	1369252	1373733	4481	145	yes	
	17	2009	346810	0.03	scaffold_5	3366779	3373770	6991	3027	yes	2009
		2017	Ep155_U_T00006866_1	0	scaffold_5	3366769	3374058	7289	873	yes	
	18	2009	260426	0.31	scaffold_5	570069	572567	2498	1919	no	2017
		2017	Ep155_U_T00006108_1	0.04	scaffold_5	570975	573657	2682	1080	yes	
	19	2009	94097	0.03	scaffold_6	279282	280501	1219	110	no	2017
		2017	Ep155_U_T00007212_1	0.01	scaffold_6	279282	280496	1214	157	yes	
	20	2009	322230	0.35	scaffold_4	3221369	3222209	840	/	/	2017
		2017	Ep155_U_T00005405_1	0.01	scaffold_4	3220823	3222517	1694	972	yes	
NO_GENE	22	2009	65946	1	scaffold_1	168020	168910	890	352	no	2017
	23	2009	241925	1	scaffold_1	2700456	2702059	1603	750	yes	2009
	24	2009	75444	1	scaffold_11	865322	866791	1469	1195	no	2017
	25	2009	334967	1	scaffold_10	685961	687176	1215	430	no	2017
	26	2009	71384	1	scaffold_2	1034513	1035594	1081	777	no	2017
	27	2009	68001	1	scaffold_4	1226218	1227846	1628	525	no	2017

Table 1. 81.48% of tested genes were demonstrated to support 2017-version annotation.

Fungal response to virus infection. One area of limited understand is how the fungal colony responds to replicating RNA. At a recent meeting (Fungal Genetics Conference, 2017), we discussed recent evidence from *Fusarium* that indicated a role for a newly identified protein

they called FD1. This protein was elevated in expression in the presence of virus, and deletion of the gene encoding it resulted in virus-like symptoms in the absence of virus. We have now identified a similar gene from *C. parasitica* and are pursuing a similar strategy to see if there is a conserved response mechanism that fungi use for combating virus infection, independent of the RNAi pathway previously identified by the Nuss lab.

Ongoing projects.

- Vegetative compatibility and Vib-1
- Genome reannotation
- Genes important for pathogenicity
- Fungal response to virus infection

Didi Ren, Mississippi State University

LysM proteins and *C. parasitica* virulence. By examining genome sequence data, *C. parasitica* was found to contain five putative proteins containing LysM motifs (2014 report). These motifs have been recognized using information from the organism's genome portal. Of relevance to this study is the potential of these proteins to act as an effector protein, which plays a role in the virulence of certain pathogens. Recent findings provided evidence of LysM containing proteins in two other fungal plant pathogens, *Cladosporium fulvum* and *Magnaporthe oryzae*, which are secreted during the initial fungal infection of the plant. It has been determined that these LysM containing proteins are able to bind to chitin, competing with the plant's pattern recognition receptors, therefore helping to overcome the host's defense response. Knockouts of four of these genes have been created, but only one showed significant reduction of virulence, a phenotype also coupled with a strong vegetative growth defect. However, one, called LM12, when eliminated, resulted in a modest increase in virulence (2015 report). Further analysis of this strain appears to show that the cell volume of the knockout is increased, although this preliminary data requires confirmation. Additional studies planned include development of mutantions in LM12 that will prevent glycosylation to test whether this modification is important for the protein's role, and to identify potential roles for the other LysM proteins in fungal behavior.

Donald Nuss, West Virginia University, adjunct

One-year study of hypovirus transmission by engineered super donor strains into a vegetative incompatibly diverse natural population of the chestnut blight fungus *Cryphonectria parasitica*. We recently reported the development of strains of the chestnut blight fungus *Cryphonectria parasitica* with enhanced ability for strain-to-strain transmission of virulence-attenuating hypoviruses (Zhang and Nuss, 2016, PNAS 113, 2062-2027). This was accomplished by systematic disruption of genes that regulate the vegetative incompatibility (*vic*) fungal allorecognition system to remove restrictions to mycovirus transmission. The results of laboratory transmission studies predict that the SD formulation could circumvent *vic* imposed restrictions to virus transmission by serving as an effective vector to introduce hypovirus into field strains representing all possible *vic* genotypic combinations of the six defined diallelic *vic* genetic loci. We will report the results of a one-year field study to test this prediction in a forest setting.

Three plots were established on the Savage River State Forest near Grantsville, MD within a clearcut stand containing an abundance of diseased American chestnut (*Castanea dentata*) root sprouts symptomatic for chestnut blight. American chestnut stems with up to three *Cryphonectria parasitica* cankers, each being less than 75% of the stem's circumference, were selected within each plot. The following number of trees and cankers were included for each plot: Plot 1: 19 trees/41 cankers; Plot 2: 17 trees/33 cankers; and Plot 3: 18 trees/31 cankers.

C. parasitica strains used in this study included *vic* genotype tester strains (Cortesi and Milgroom, 1998) EU5 (ATCC MYA-1048) and EU6 (ATCC MYA-1049) and the super mycovirus donor strains SD328 and SD82 (Zhang and Nuss, 2016, PNAS 113, 2062-2027). The convention used to describe the *vic* genotype of *C. parasitica* strains specifies which allele, designated 1 or 2, is present at the six defined diallelic *vic* genetic loci. For example, the *vic* genotype for the *C. parasitica* reference strain EP155 is *vic1-2*, *vic2-2*, *vic3-1*, *vic4-1*, *vic6-2* and *vic7-2* (abbreviated 2211-22). The *vic* genotypes for strains EU5 and EU6 are respectively 2211-22 and 2111-22, while the *vic* genotypes for SD strains DS328 and DS82 are respectively ~~2~~2~~1~~1-~~22~~ and ~~2~~1~~1~~1-~~22~~, where the strikes indicate a gene disruption at the respective *vic* loci. For this study, the EU and SD strains were infected with hypovirus CHV-1/EP713.

Each treatment was randomly assigned to one of three designated plots. Cankers in Plot 1 (SD plot) were treated with a combination of CHV-1/EP713-infected strains SD 328 (~~2~~2~~1~~1-~~22~~) and SD 82 (~~2~~1~~1~~1-~~22~~). Plot 2 (EU5/6 plot) cankers were treated with the combination of CHV-1/EP713-infected strains EU5 (2211-22) and EU6 (2111-22) that have the same *vic* genotype as SD328 and SD82 without the gene disruptions. Plot 3 (sham plot) received treatments with an agar slurry without any fungal inoculum to serve as a negative control.

Cankers were measured and subjected to a one-time treatment in July 2016. New cankers were identified in September and November of 2016, measured, sampled and treated with the plot-appropriate inoculum. Post-treatment measurements and sampling of the initial July 2016-treated cankers and the September 2016-treated new cankers was performed in November 2016 and again in July 2017, while post-treatment sampling of new cankers treated in November 2016 was performed in July 2017. The results of the study demonstrated efficient hypovirus transmission by engineered super donor strains into a vegetative incompatibly diverse natural population of the chestnut blight fungus *Cryphonectria parasitica*.

Cameron Stauder, West Virginia University

Characterizing vegetative incompatibility gene profiles of *Cryphonectria parasitica.*
Cameron Stauder, D.L. Nuss, W.L. MacDonald, M.T. Kasson. Debilitating hypoviruses of *Cryphonectria parasitica* are horizontally disseminated via hyphal anastomosis between vegetatively compatible strains. Hyphal anastomosis for *C. parasitica* is regulated by a vegetative incompatibility (*vic*) system comprised of six diallelic loci, providing for 64 unique *vic* genotypes. Strain-to-strain dissemination of hypoviruses among a wild *C. parasitica* population is therefore restricted by the population's *vic* genotype diversity. Here, we present an extensive survey of the *vic* diversity of infections on wild American chestnuts (*Castnea dentata*) in the Savage River State Forest near Grantsville, MD. Approximately, 190 cankers were sampled over 1.5 years. The *vic* genotype of associated *C. parasitica* strains was resolved using a multilocus PCR protocol described by Short et al. (2015). In total, 37 of the 64 possible *vic* genotypes were

detected using this method. Multiple (3 to 5) strains were characterized for 16 of the 190 cankers, and 75% of that subset were found to be comprised of multiple *vic* genotypes thus providing evidence of canker complexity. Levels of *vic* genotype diversity within a canker and across a population, such as those presented here, likely determine the success of hypovirus transmission and dissemination.

Treatment delivery of superdonor fungus. A.M. Metheny, D.L. Nuss, M.T. Kasson and W.L. MacDonald. The purpose of this project is to assess how treatment type and hypovirus can impact efficacy of the superdonor strain of *Cryphonectria parasitica* when used to preserve the longevity of American chestnut stems. Fifty trees were selected, most of which were free of cankers, at a site in the Savage River State Forest near Grantsville, MD. For those trees completely free of infection, three artificial cankers were created, at 50 cm, 100 cm and 150 cm with a virulent isolate (EU 12). For those trees with one natural canker, only two artificial cankers were established. Of the 161 cankers in the study, 29 are natural and 132 are artificial. The natural infections were sampled and genotyped. Cankers were initiated on 22 June 2017. By 8 August 2017, the average canker size was 6 cm (length) by 4 cm (width). The three treatments included: (1) scratch (a beehive comb was used to scratch through the bark of the canker to the cambium layer and inoculum spread over the wounds); (2) punch (a leather punch was used to create circular wounds around the canker margins and interior which are then filled with inoculum); and (3) paint (inoculum was spread on the canker using a paintbrush without wounding the stem). The efficacy of two CHV-1 hypoviruses also will be compared, Euro 7 and EP 713. Inoculum will be comprised of a mixture of either SD 328 (713) and SD 82 (713) or SD 328 (Euro 7) and SD 82 (Euro 7). All cankers on a tree were treated identically, with the same treatment method and hypovirus, and the trees were randomly selected for each treatment. Trees will be measured periodically until August 2018 (one-year anniversary) at which time all cankers will be measured. In addition, cankers will sampled to assess hypovirus transmission. Resulting isolates will genotyped according to the method of Short et al. (App. Env. Micro. 2015. 81:5736-5742) to assess *vic* genotypes of virulent and hypovirulent isolates.

Mark Double, West Virginia University

Introduction of hypoviruses at West Salem, Wisconsin (in cooperation with D.F. Fulbright and A.M. Jarosz, Michigan State University; and, A. Davelos Baines, University of Wisconsin-La Crosse). The stand of American chestnut in West Salem became infected with chestnut blight in the late 1980s after 100 years of blight-free growth. Hypovirus introduction (individual canker treatment) was conducted from 1992-1997 (700 cankers on 133 trees received inoculum). From 1998-2002 hypovirus introduction was halted. In 2001, due to a large increase in the number of cankers in the stand, twelve permanent plots were established in three regions of the stand representing differing levels of disease: Disease Center; Front; and, Beyond the Front. Hypoviruses were reintroduced in 2003; annual treatment has continued through 2015. Approximately 25% of the trees in each plot are untreated to assess tree-to-tree spread of hypovirulent strains.

Hypovirus spread has been assessed annually by analyzing isolates of *C. parasitica* that arise from bark samples. Hypovirulent isolates are recovered most readily from treated cankers followed by non-treated cankers on treated trees. Hypoviruses have spread less effectively to

untreated trees. Since 1992, a total of 3,817 cankers have been identified in the 12 plots. Two hundred, forty cankers on living trees were sampled in July 2016; 98 were newly discovered.

General observations:

- When the 12 permanent plots were established in 2001, there were 517 living stems included in the study. As of 2016, 54% of the original stems in the Disease Center plots remained alive compared to 24% and 8% in the Disease Front and Beyond the Disease Front plots, respectively. Some loss of stems may be attributed to the harsh winters of 2013-14 and 2014-15.
- Vegetative compatibility type WS-1 continues to be the dominant vc type in the stand although its frequency gradually has decreased from 100% in 1995 to 80% in 2016. WS-2 and WS-3 were found at rates of 5% and 9%, respectively.

Matt Kolp, Michigan State University

Intra-canker variability for secondary fungi. (In cooperation with William MacDonald and Mark Double, West Virginia University). The fungal community of chestnut blight cankers in surviving chestnut populations in northern Michigan and in West Salem, Wisconsin contains virulent (CP) and hypovirulent *C. parasitica* (HCP) along with dozens of other fungal taxa (collectively Non-CP). We predict that cankers that girdle a stem or branch (girdling canker) and those that do not (non-girdling canker) are different with respect to the composition and distribution of fungi within a given canker type (Figure 1).

A K-means cluster analysis reveals four distinct fungal community types within sampled cankers with variable relative proportions of 61 different fungal taxa (Figure 2). Using a mixed model, we show that fungal community clusters have different probabilities of being associated with girdling and non-girdling chestnut blight cankers (Figure 3). A high frequency of HCP (cluster B) or Non-CP (cluster A) within a canker was associated with high probabilities of being a non-girdling canker (Figure 3). CP was the dominant fungus in clusters C and D, which were more commonly associated with girdling cankers (Figure 3).

The distribution of CP, HCP, and Non-CP within cankers was less spatially structured than predicted (Table 1). Instead, the distribution within cankers is more of a mosaic, with CP, HCP, and Non-CP occurring in all areas of cankers. This may indicate that canker dynamics are the result of complex interactions between Non-CP and virulent (CP) and hypovirulent (HCP) forms of the blight pathogen *C. parasitica*.

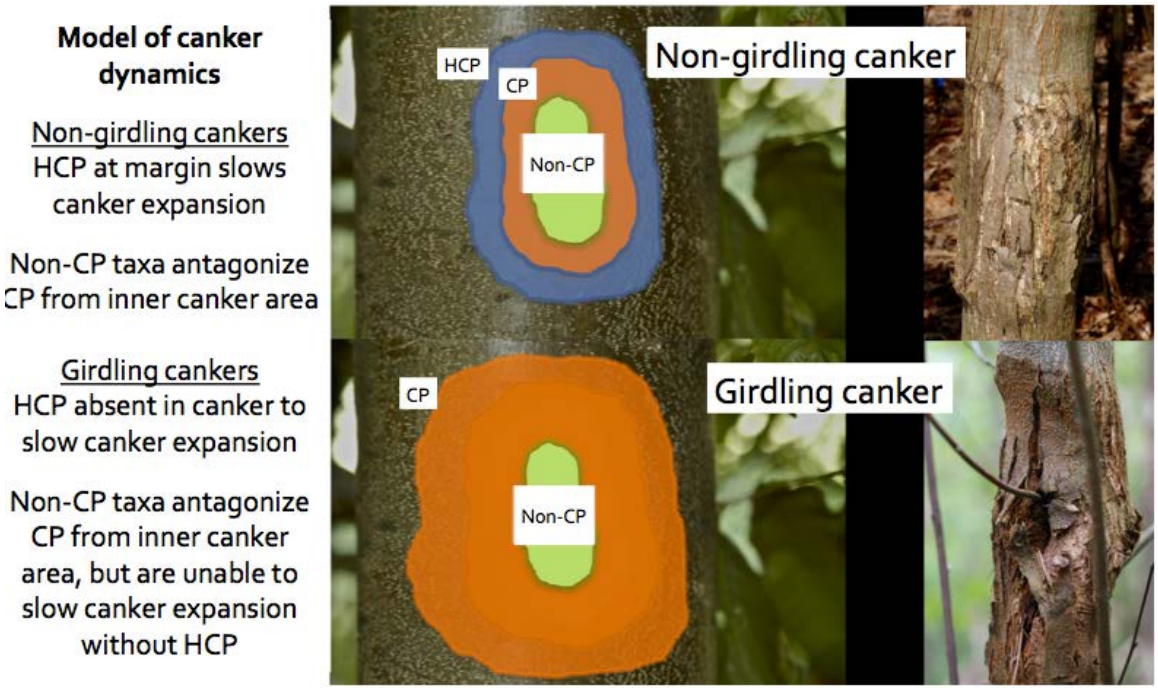

Figure 1: Cankers that fail to girdle a stem (**top**) are predicted to have hypovirus-infected *C. parasitica* (HCP) concentrated along the canker's margin, the uninfected pathogen (CP) in the middle, and secondary fungi (Non-CP) invading the inner area of a canker. Cankers that expand rapidly around a stem and girdle trees (**bottom**) will have abundant CP at the margin and Non-CP invading the inner area but far from the margin.

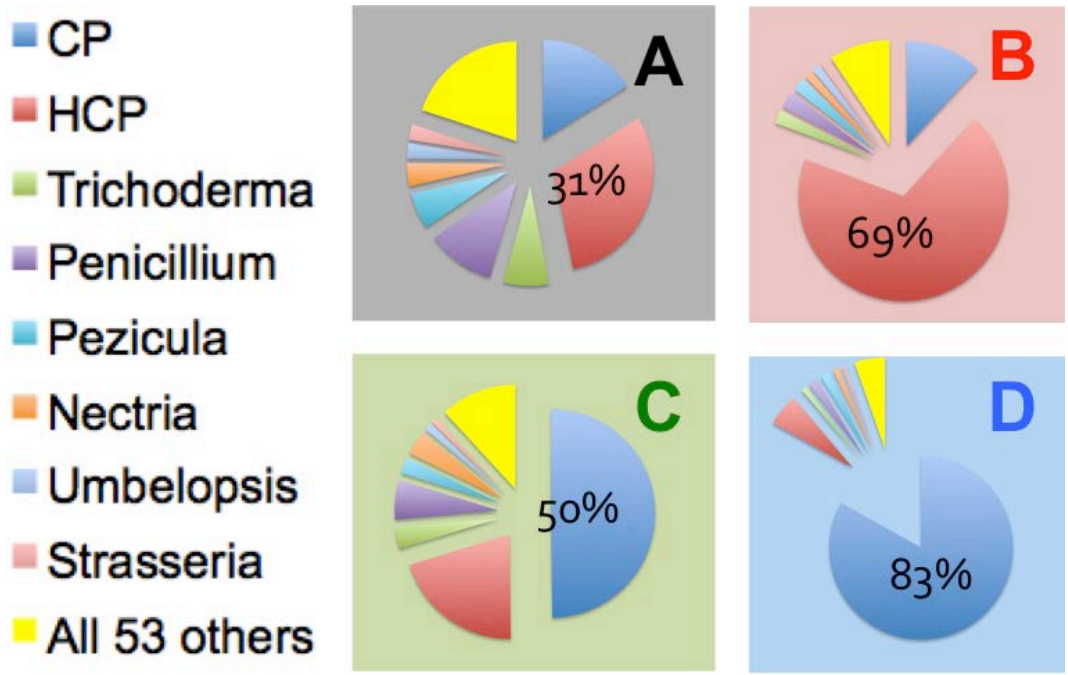

Figure 2: Frequency of fungal taxa within four representative community clusters (as defined by K-means clustering analysis). The percentage of the most abundant taxa in each cluster is shown.

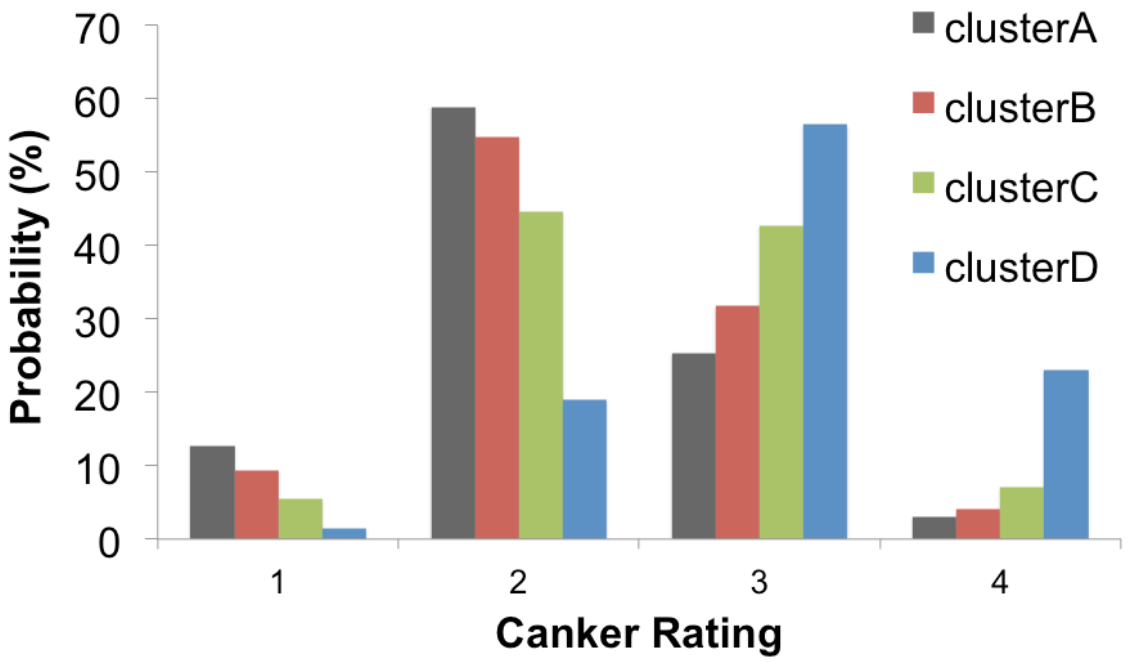

Figure 3: Community clusters and their association with non-girdling (canker rating 1 or 2) and girdling (canker rating of 3 or 4) cankers.
Data based on all years and across all populations.

Table 1: Distribution of CP, HCP, and Non-CP sampled within the margin (edge of chestnut blight canker) and inner area of non-girdling and girdling cankers from Michigan and West Salem, WI chestnut populations during 2012-2016.

	Non-girdling Cankers		Girdling Cankers	
	margin	inner	margin	inner
CP	24.7*	23.3	46.2	39.4
HCP	36.0	37.6	26.3	31.7
Non-CP	39.3	39.1	27.5	28.9

* = Frequency of isolates across all years and populations

<u>OBJECTIVE 3.</u> To investigate chestnut reestablishment in orchard and forest settings with special consideration of the current and historical knowledge of the species and its interaction with other pests and pathogens

Scott Schlarbaum, University of Tennessee

Research by the UT Tree Improvement Program (UT-TIP) continued to be in a supporting role for two USDA Forest Service scientists, Dr. Stacy Clark, Southern Research Station, and Dr. Leila Pinchot, Northern Research Station. Chestnut seedlings are processed through the UT-TIP facilities and planted and/or evaluated with assistance from UT-TIP staff in consultation with Dr. Schlarbaum (biology) and Dr. Arnold Saxton (statistics).

Stacy Clark, USDA-FS, Southern Station

We are continuing to follow eleven of thirteen plantings established to test the blight resistance, nursery seedling quality, and competitive ability of advanced breeding material from The American Chestnut Foundation and the Connecticut Agricultural Experiment Station. Results show that the BC_3F_3 generation is more resistant than the American chestnut (*Castanea dentata*), less resistant than the Chinese chestnut (*C. mollissima*), and is similar to the BC_1F_3 and BC_2F_3 generations. Seedling size at the time of planting improved competitive ability, total height and diameter growth, but the largest seedlings had slightly lower survival. Root rot from *Phytophthora cinnamomi* and repeated deer browsing hindered seedling success. Seedlings could be successfully competitive on high quality sites to the eighth growing season if they were relatively high quality at the time of planting, protected from deer browse, and were not negatively impacted by Phytophthora disease.

Leila Pinchot, USDA-FS, Northern Station

2015 Study on effects of site quality on competitive ability and blight resistance. The study was established to assess the ability of American, Chinese, and backcross chestnut trees to compete with woody vegetation and resist blight infection across a range of sites ranging in moisture availability. Seedlings were planted on 15 sites, categorized as mesic, xeric, and intermediate, within deer exclosures. At the end of each growing season, the height and ground-level diameter of the planted seedlings and the height and species of the tallest woody competing seedling within 1.3 m of each chestnut is recorded. Survival and growth have been excellent with relatively low incidence of blight. Results will guide site selection for American chestnut reintroduction plantings.

Additional Collaborators: USDA Forest Service, Northern Research Station (Alex Royo, Matt Peters) and Connecticut Agricultural Experiment Station (Sandra Anagnostakis).

2017 Chestnut Silviculture Study. This study will evaluate long-term (10+ year) survival and growth of chestnut seed and seedlings planted in each of the three stages of the three-stage shelterwood harvest system commonly used to regenerate oak in the Allegheny plateau region of northwestern Pennsylvania. Seedlings from The American Chestnut Foundation's and Connecticut Experiment Station's breeding programs were planted on nine treatment sites on the Allegheny National Forest. Three replicates each of three silvicultural treatments used to regenerate oak were used in this study. All chestnuts are protected from browsing by five foot-

tall tree shelters. Height and ground-level diameter of the planted seedlings and the height and species of the tallest woody competing seedling within 1.3 m of each chestnut will be recorded at the end of each growing season.

Additional Collaborators: Allegheny National Forest (Scott Tepke, Greg Stanford), the Connecticut Agricultural Experiment Station (Sandra Anagnostakis), and The American Chestnut Foundation.

Andrew Jarosz and Dennis Fulbright, Michigan State University

Commercial chestnut orchards. The best year in orchard chestnut production since this program was started was 2016. The chestnut growers' cooperative, Chestnut Growers, Inc. which represents about 30 growers, produced and marketed the largest number of chestnuts in their history (established 2001). Over 212,000 pounds of chestnuts were produced and marketed. By far, the European X Japanese cultivar 'Colossal' produced the most chestnuts for marketing by the cooperative. Three farms produced over 5,000 pounds/acre of 'Colossal' chestnuts. The MSU cultivar trial established at Clarksville, Michigan, yielded chestnuts matching the 5,000 pounds/acre when yield was extrapolated for acreage. Many of these farms have their 'Colossal' trees treated for chestnut blight with the hypovirulent slurry developed at Michigan State University based on hypovirus, CHV3-GH2. For the state of Michigan, more than 15,000 grafted European X Japanese cultivars have been planted in orchards by Michigan growers. Newer European X Japanese cultivars like 'Marigoule' and 'Marsol' are supposed to be chestnut blight tolerant, but this has not been tested in Michigan.

Michigan chestnut census. The 22th census of six American chestnut populations was completed in August 2017. Two recovering populations, County Line and Roscommon, continue to exhibit strong tree survivorship and growth. Recruitment in 2017 was average. Trees at the Frankfort, Leelanau, Missaukee sites all continue to decline due to the ravages of chestnut blight. No seed production has occurred at Missaukee sites since 2012. Unfortunately, one of the Missaukee study sites was logged so trees at this site were not monitored in 2017. We will return in the spring of 2018 to determine if enough trees survived the timber operations.

Laurel Rodgers, Shenandoah University

Is Illumina sequencing useful for studying mircobiomes in trees? Summary. The long-term goal of this project is to determine if there are different fungal endophyte communities living in chestnut trees resistant to *Cryphonectria parasitica* (Chinese chestnuts and chestnut hybrids) compared to chestnut trees that are not resistant to *C. parasitica* (American chestnuts). Endophytes are fungi that grow within a plant without causing harm and likely prevent colonization of pathogenic fungi. Endophytes that provide resistance against *C. parsistica* can be used as a biocontrol against the fungus, assisting in the reestablishment of the American chestnuts into our forests. We identified fungi growing within chestnut trees using traditional Sanger sequencing and Illumina DNA sequencing. After comparing the data from both methods, we concluded that traditional Sanger sequencing is the most reliable method for us to use to study endophyte populations within chestnut trees.

Introduction. Endophytes are defined as fungi and bacteria living within a plant without causing harm. Surveys within the last thirty years have found a surprising number of fungi growing in healthy trees. For example, an average 11.5 species of fungi were isolated from leaves and twigs of the American hornbeam (*Carpinus caroliniana)* located in New Jersey and West Virginia (Bills 1991). In another study, Danti *et al* (2002) isolated forty-four different fungal species from ten European birch (*Fagus sylvatica*) trees growing within a 200m-diameter area. Despite the fact that fungi have been found in living tissue of all plants surveyed to date, fungal endophytes represent a significantly understudied, and poorly understood, component of our ecosystem (Porras-Alfaro and Bayman 2011; Bacon and White 2000).

Like the bacterial microbiome in the human body, endophytes in a tree are likely to play a significant role in protection from pathogens. For example, fungal endophytes may outcompete pathogenic fungi within a tree. Arnold *et al* (2003) investigated the leaf mortality of *Theobroma cacao* seedlings after being inoculated with six *Theobroma cacoa* endophytes and then challenged with a pathogenic fungus. The seedlings inoculated with endophytes had a significantly lower rate of leaf mortality compared to control seedlings that were not inoculated prior to the administration of the pathogenic fungus. While some endophytes do stimulate systemic defenses within a plant, it is likely in this case that the introduced endophytes were inhibiting the pathogen through direct interactions.

Several endophytes are also known to secrete anti-microbial chemicals, which are likely to inhibit the growth of pathogenic fungi. *Acremonium sp.* naturally grows on the European yew (*Taxus baccata)* and inhibits the growth of fungi in *in vitro* studies. *Acremodium sp.* produces the anticancer and antifungal peptide leucinostatin A. Unlike many plants, the yew tree has no adverse reaction to the presence of leucinostatin A. Based on their study, Strobel and Hess *et al* (1997) hypothesize that *Acremodium sp.* protects the yew tree from colonization by pathogenic fungi. In another example, *Muscodaor albus* produces a combination of volatile antimicrobial compounds that inhibit the growth of other fungi on the cinnamon tree (*Cinnamomum zelanicum*) (Strobel et al. 2001). Improving our understanding of natural endophyte populations and how they may vary between tree species and between geographical locations will be valuable when combating the spread of devastating diseases throughout our forests.

The American chestnut (*Castanea dentata*) tree is just one species that may benefit from the enhancement of natural endophyte populations. Prior to the early 1900s, the American chestnut was the dominant tree species within East coast US forests. The tree was a keystone species both economically and environmentally. Unlike the dominant species today, the oak and the hickory, the American chestnut provided a reliable supply of nuts every year. The high tannin content within these trees also supported a large leather tanning industry and its wood was highly valued for timber because it resists decay without the chemical treatment needed for today's common timber trees. At the turn of the 20th century, the fungus *Cryphonectria parasitica* was accidently introduced to the United States. Unlike the Chinese chestnut (*Castanea mollissima*), the American chestnut has no resistance to *C. parasitica*. By the middle of the 20th century, nearly all American chestnuts had been wiped out from our forests (Anagnostakis 1987; Griffin 2000).

The goal of this project is to compare the normal endophyte populations growing in the American chestnut to those in the Chinese chestnut and chestnut hybrid trees that are resistant

to *C. parasitica*. Due to chemical differences within each tree, it is reasonable to hypothesize that different endophyte species inhabit each species. These differences will provide further clues that indicate why the Chinese chestnut tree is more resistant than the American chestnut. This study will allow us to identify an endophyte, or a group of endophytes, that grow on resistant chestnut trees, but not in American chestnut trees lacking resistance. These endophytes can then be assessed for use in the development of biocontrol methods against *C. parasitica* in future American chestnut reforestation projects.

Summary of Methods. In June 2016, we collected 10 samples each from one American chestnut and one Chinese chestnut tree growing side by side in plot B at Blandy Experimental Farm (Boyce, VA). These trees were 7 years old at the time of sample collection. All but one sample was collected from healthy tissue on the main stem or a large branch. The one remaining sample was collected from a canker as a positive control. Two additional samples were isolated in September 2016 from canker tissue on the American chestnut tree. At each sample site one 3/16th inch plug was removed for Illumina sequencing and one 3/16th inch plug was removed for growing, isolating, and sequencing fungi with traditional Sanger sequencing. Prior to isolation, the bark was surface sterilized with 70% ethanol. After isolation, each plug was placed in a sterile 1.5ml centrifuge tube for transport to the lab.

Each sample used for Sanger sequencing was placed in nutrient free 1.5% agar plates and observed daily for a week. Hyphae growing from the samples were removed and placed on 3.9% potato dextrose agar plates. Each fungal sample was grown for 3-5 days and then transferred to a new plate in order to confirm a pure sample was present on the agar plate. DNA was isolated from hyphae by using a Qiagen DNeasy plant mini kit (Qiagen, Hildon, Germany). The fungal ITS region, the DNA region between the large and small ribosomal subunit genes, was then amplified by PCR using ITS 1 (TCCGTAGGTGAACCTGCGG) and ITS 4 primers (TCCTCCGCTTATTGATATGC). PCR samples were cleaned using a QIAquick® PCR Purification Kit (Qiagen, Hildon, Germany) and submitted to Euruofins Genomics (Louisville, KY) for Sanger sequencing. Fungal identity for each sequence was determined using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the UNITE database (https://unite.ut.ee/).

Five samples from each tree were selected for Illumina based on the number of fungal species isolated from the sample. After collection, samples for Illumina sequencing were ground into a powder using liquid nitrogen prior to isolating DNA using a Qiagen DNeasy plant mini kit. Isolated DNA was sent to the UNC Genomics Core Facility (Chapel Hill, NC) for Illumina sequencing. DNA was prepared for Illumina using the facility's in-house protocol for the fungal ITS2 region. The collected Illumina data was prepared using the same parameters as Bálint et al. (Bálint 2014) and provided as raw data, BLAST files and text files.

Results and Discussion. The first question investigated in this study is whether the fungi found by traditional isolation and sequencing could also be identified by Illumina sequencing. Table 1 lists the fungi isolated and identified in each of the tree samples that were used for both traditional sequencing and Illumina. All samples identified by an "AC" were from the American chestnut and all samples identified by a "CC" are from the Chinese chestnut. In general, the higher the number of sequences found by Illumina the greater the prevalence of a species living within the sample collected. As seen in Table 1, only two fungal cultures, *Monochaetia* (in AC 14) and *Hypoxylon rubiginosum* (in AC 13) that were not identified by both Sanger and Illumina sequencing methods. Four samples, *Sordariomyces*, *Pestalotiopsis*

microspore, *Lecythophoa*, and *Biscogniauxia mediterranea* had fewer than 100 reads by Illumina sequencing. Of the remaining species identified by Sanger sequencing, five had fewer then 600 reads, and five species had more than 1000 reads. Due to the wide range of Illumina read numbers seen in this data set, we can conclude that there is no direct correlation between ability to culture a fungus from a sample and the ability for it to be identified by Illumina sequencing.

Table 1: Comparison of fungi found by Sanger sequencing and Illumina sequencing within a single tree sample

Tree number	Fungus species identified by traditional sequencing	Number of reads by Illumina for each fungus
AC13	*Sordariomyces* genus	21
	Hypoxylon rubiginosum	24 for genus, 0 for species
AC14	*Pestalotiopsis microspora*	47
	Diplodia seriata	1659
	Leptosphaerulina chartarum	209
	Monochaetia genus	0
AC15	sequencing results were not conclusive	
AC16	*Lecythophora* genus	60
AC17	*Pestalotiopsis microspora*	427
CC4	*Fusarium Poae*	4087 for genus, 0 for species
CC5	*Biscogniauxia mediterranea*	58
CC7	*Epicoccum nigrum*	3351
	Pestalotiopsis microspora	609
CC8	*Epicoccum nigrum*	3634
CC9	*Fusarium (equiseti* or *verticillioides)*	5105
	Alternaria alternata	275
	Epicoccum nigrum	3325
CC11	*Pestalotiopsis microspora*	574

We next asked whether fungi identified by traditional methods in one tree sample were identified by Illumina sequencing in the other tree samples. Table 2 shows the frequency with which each fungus was identified by Illumina sequencing in each tree sample. The highlighted squares indicated the samples in which the fungus was also identified by Sanger sequencing within that tree sample. For the purposes of this table, we only included fungi in which both the genus and species were identified by Sanger sequencing. The majority of the samples cultured in our lab were identified by Illumina sequencing in all of the tree samples. The two exceptions were *Hypoxylon rubiginosum* and *Fusarium poae*. *Alternaria alternate* had very low frequency of identification in two tree samples. Of interest, *Hypoxylon rubignosum* was cultured from sample AC13, but was only identified by Illumina in three of the Chinese chestnut samples. I am concerned that Illumina sequencing is identifying species in the Chinese chestnut tree that we were only able to culture from the American chestnut tree and vice versa, indicating that Illumina may identify an unacceptable number of false positives within the results.

Table 2: Number of sequences found by Illumina in each tree sample for each fungus identified by traditional sequencing.

	Number of reads by Illumina in each tree sample									
Fungus Name	**AC13**	**AC14**	**AC16**	**AC17**	**CC4**	**CC5**	**CC7**	**CC8**	**CC9**	**CC11**
Hypoxylon rubiginosum	0	0	0	0	0	0	137	0	113	60
Diplodia seriata	1793	1659	1647	1753	1723	1559	1717	1659	1661	1655
Leptosphaerulina chartarum	360	209	92	495	597	602	456	609	156	161
Fusarium poae	0	4	2	0	0	0	1	0	0	0
Alternaria alternata	82	130	54	5	121	142	473	544	275	1
Biscogniauxia mediterranea	0	0	1	0	16	58	0	0	222	1
Epicoccum nigrum	3279	2967	2403	1845	3415	3097	3561	3634	3325	3418
Pestalotiopsis microspora	63	47	33	427	607	602	609	609	609	574

In order to determine if Illumina was providing false positive results, we visually scanned the Illumina results for species that were unlikely to be growing within our chestnut trees. Table 3 provides a list of six organisms identified in our samples by Illumina sequencing that I believe are unlikely to be growing within the chestnut trees we sampled. *Cystobasidium ongulense* is a species of yeast that has been found growing in the artic and has specialized features that allow it to survive at freezing and near freezing temperatures (Tsuji 2017). *Sakaguchia dacryoidea* is a marine yeast that has been found in various marine environments around the world, including the Indian, Pacific, and Antarctic oceans (Fell 2011). *Peudogymnoascus destrucatans* causes white nose disease in bats and is typically found in cold environments such as caves (Reynolds 2015). *Dipodascus tetrasporeus* is a yeast that has been found in deep sea sediments in the Pacific Ocean near Japan (Nagahama 2008). *Geopora arenicola* is a fungus that grows in dry, sandy soils in Europe (Tao and Spooner 2003). *Exophiala dermatitidis* is a species of fungus that is most frequently found in wet, warm environments (such as incubators and steam baths) and has rarely been found in nature (Sudhadham 2008). Each of these species is very unlikely to be growing within our chestnut trees and most were identified by Illumina at a high frequency. This set of data leads me to conclude that the number false positives generated by Illumina sequencing from our chestnut tree samples will make analyzing the data and accurately comparing of the microbiomes of the two tree species very difficult.

Table 3: List of species found by Illumina within chestnut trees that are unlikely to be living within the trees.												
		Number of hits within each tree sample										
Species	**Reason Unlikely**	AC 13	AC 14	AC 15	AC 16	AC 17	CC 4	CC 5	CC 7	CC 8	CC 9	CC 11
Cystobasidium ongulense	species of yeast found in the arctic	783	720	531	511	783	778	360	783	783	783	783
Sakaguchia dacryoidea	marine yeast	348	288	161	56	339	327	127	348	348	348	284
Pseudogymnoascus destructans	fungus that causes disease in bats	289	63	2115	2299	223	2629	289	5076	1098	3149	4235
Dipodascus tetrasporeus	yeast found in deep sea vents	0	0	1	0	0	5	83	1	41	87	41
Geopora arenicola	only found in soils in Europe	0	0	403	0	0	878	668	609	197	726	0
Exophiala dermatitidis	rarely found in nature, usually found in warm, wet environments	0	0	3223	0	0	1563	312	694	876	1	2

For our final analysis, we used the traditional sequencing data to compare the microbiomes of the two sampled chestnut trees. Table 4 lists the fungi cultured and identified from the American chestnut tree only, the Chinese chestnut tree only, and both trees. There are more fungi included in this list than in the previous tables because all samples were included for this table, and not just the samples that were also used for Illumina sequencing. Despite the fact that they were growing next to each other within the research plot, there were only two fungi species cultured from both the American and the Chinese chestnut trees. Seven species of fungi were cultured only from the American chestnut and six species were cultured only from the Chinese chestnut tree. We cannot draw any major conclusions from this data because we only sampled one tree of each species, however, it is still interesting that these initial data indicate that there may in fact be differences in the microbiomes of the American and Chinese chestnut trees.

Table 4: List of species cultured and identified from Chestnut tree.

Fungi Cultured from American Chestnut	**Fungi Cultured from Chinese Chestnut**	**Fungi Cultured from Both American and Chinese Chestnut trees**
Sordariomyces (species not clear)	*Fusarium Poae*	*Pestalotiopsis microspora*
Hypoxylon rubiginosum	*Biscogniauxia mediterranea*	*Epicoccum nigrum*
Diplodia seriata	*Phomopsis vaccinii*	
Leptosphaerulina chartarum	*Fusarium (equiseti* or *verticillioides)*	
Cladosporium cladosprioides	*Alternaria alternata*	
Lecythophora (species not clear)	*Penicillium corylophilium*	
Monochaetia dimorphospora		

Conclusions. Based on the results of this study, we have concluded that Illumina sequencing is not the best method for comparing the fungal microbiomes growing within chestnut trees for the following reasons:

1. Six of the fungal species cultured from the trees were identified by Illumina with a very low frequency.
2. The fungal species that were cultured only from one species of tree, were also identified by Illumina in the other species of tree, indicating the possibility of false positive results.
3. There were at least six fungal species identified by Illumina that are highly unlikely to be growing within our chestnut trees, further confirming the likelihood of false positives being identified by Illumina.
4. We were unable to collect Illumina data from the canker location on the American chestnut tree. While the purpose of this project is to look at fungi growing in healthy tissue, it would be useful in the future to be able to compare the fungal community in healthy tissue to infected tissue.

Fred Hebard, The American Chestnut Foundation, Meadowview

Species identification in *Castanea* by staining. Hebard examines glandular hairs on dried leaves using a compound microscope. Glandular hairs are more prevalent on the lower leaf surface. Hebard showed examples of Japanese chestnut hairs that had 8 cells (lobes) compared to American chestnut hairs that resemble hot-cross buns. Hebard noted that American hairs are smaller than those on Japanese chestnut. Hebard showed hairs from American chestnut and Allegheny chinkapin using a scanning electron microscope. Allegheny chinkapin hairs have two types of hairs: stalked (cigar-shaped) and hot cross buns. Hebard used whole tissue mounts for observation in transmitted light. The tissues are cleared and the cytoplasmic contents are rendered invisible. Herbarium specimens are often decolored prior to clearing. Because glandular hairs are small, decolorizing proved necessary only for chestnut. Decolorizing is conducted by soaking in a 10% bleach solution. Tissue processing included:

- Squares measuring 2-3 mm on a side were cut from the interveinal portion of leaves.
- Specimens were placed in 1/8 ounce ointment vials in bleach overnight.
- Specimens were rinsed four times in distilled water.
- Stains were applied for 10 minutes followed by 4 rinses in distilled water.
- Specimens were mounted abaxial side up in a 1:1 glycerol:water solution.

A 0.5% solution of alcian blue does not work well with fresh tissue. A 0.1% solution of safranin O always worked but it can diffuse into all tissues and strain everything red.
Conclusions:

- Staining can be implemented with minimal lab facilities common to most biologists.
- Chemicals are reasonably non-toxic.
- A large number of hairs can be assessed quickly.
- The sample throughput is high.

Sandra Anagnostakis, The Connecticut Agricultural Experiment Station

Hypovirulent strains distributed. After receiving permission from the USDA in 1972, hypovirulent strains of *Cryphonectria parasitica* were sent out to people all over the U.S. When H strains are recovered now in new locations, checking the site against this list may reveal their origin. I have mailing addresses for most of these, should they be needed.

STATE	TOWN	PERSON	STRAINS SENT	DATE	ZIP
AR	Hot Springs	Langdon, Keith		1982	71902
AL	Florence	Moore, Jack H.	4, 43, 47, 49, 51, 60, 88, 92	1979	35630
CT	Haddam	Burton, Terry	4, 43, 47, 49, 51, 60, 88, 92	1979	06438
CT	East Hartford	Hibberd, John			06108
CT	Deep River	House, Stanley	43	1976	06417
CT	Guilford	McIntyre, John	43	1975	06437
CT	New Milford	McNeeley, John	43	1976	06776
CT	Cornwall	McNeeley, John	[M2]		
CT	Falls Village	McNeely, John	Slurry 10	1983	06031
CT	Hamden	Melillo, Lou	43	1976	06514
CT	Greenwich	Nichols, H.	[F], [I]		
CT	Branford	Nikou, Dr. Nicklas	43	1976	06405
CT	Wilton	Price, Mrs. Irwin	43	1976	06897
CT	Norfolk	Russ, Darrell	43	1976	06508
CT	North Haven	Stephens, George	43	1975	06473
CT	Killingworth	Tuckerman, David			06417
CT	Hamden	Walgren, Paul	43	1976	06518
CT	Quaker Hill	York, Edward	Slurry 10	1983	06379
D.C.	Washington	Morton, Eugene	4, 43, 47, 49, 51, 60, 88, 92, 171, 172	1980	20560
D.C.	Washington	Webster, Horace V.	4, 43, 47, 49, 51, 60, 88, 92	1979	20242
DE	Wilmington	Layton, Rodney M.	4, 43, 47, 60	1978	19801
GA	Atlanta	Schwind, Robert L.	4, 47, 49, 51, 60, 88, 92	1980	30324
IN	Richmond	Hendricks, Donald	43	1976	47374
IN	Indianapolis	Lewis, Warren	Slurry 5	1982	46240
IN	W. Lafayette	McCloud, Thomas	4, 47, 49, 51, 60, 88, 92	1981	47906
KY	Munfordville	Cloar, A. E.	14, 50, 60, 90	1977	42765
KY	Munfordville	Middleton	4, 43, 47, 49, 51, 60, 88, 92, 171, 172	1980	42765
MA	Winchester	Barker, E. Gilman	14, 50, 60, 90	1977	01890
MA	Winchester	Barker, E. Gilman	Slurry 5	1979	01890
MA	Dalton	Beebe, Larry			01226
MA	Dalton	Beebe, Larry	47, 50, 234, 420, 504, 518	1980	01226
MA	Dalton	Beebe, Larry	4, 43, 47, 49, 51, 60, 88, 92, 171, 172	1980	01226
MA	Dalton	Beebe, Larry	4, 43, 47, 49, 51, 60, 88, 92	1981	01226
MA	Dalton	Beebe, Larry	Slurry 5	1982	01226
MA	Dalton	Beebe, Larry	Slurry 10	1983	01226
MA	Longmeadow	Dreifus, David W.	47, 50, 234, 420, 504, 518	1980	01106
MA	Amherst	Holmes, Francis W.	43	1976	01002
MA	Harvard	Jorgensen, Neil	Slurry 8		01451
MA	Harvard	Jorgensen, Neil	4, 43, 47, 49, 51, 60, 88, 92, 171, 172	1980	01451

MA	Petersham	Lorimer, Craig	Bar H	1975	01366
MA	Acton	Roth, Franklin D.	14, 50, 60, 90	1977	01720
ME	Rockport	Blanchard, Peter A.			
ME		Harkness Memorial	[F], [I]		
ME	East Holden	Miller, Neil			
MI	Ann Arbor	Kaufman, Peter	2024 V, 2025 H, 2043 H	1974	48104
MI	East Lansing	Weidlich, W. H.	Bar H	1975	48824
MO	Columbia	Millikan, Dr. D. F.	67, 42 (42[HI2], 42[HM1+HM2])	1982	65211
NC	Research Triangle	Kuhlman, E. G.	3, 43, 52, 53, 54	1976	27709
NH	Walpole	Hubbard, Leslie	4, 43, 47, 60	1978	03608
NH	Walpole	Hubbard, Leslie	4, 43, 47, 49, 51, 60, 88, 92	1981	03608
NH	Walpole	Hubbard, Leslie an	[M2]		
NH	Potter Place	Powers, Henry	4, 43, 47, 49, 51, 60, 72, 88, 92, 171,	1980	03265
NH	Potter Place	Powers, Henry	4, 43, 47, 49, 51, 60, 88, 92	1981	03265
NH	Potter Place	Powers, Henry	Slurry 5	1982	03265
NJ	Morristown	Ehrenfeld, Joan	14, 50, 60, 90	1978	
NJ	Highlands	Emery, Albert	4, 43, 47, 49, 51, 60, 88, 92	1979	07732
NJ	Highlands	Emery, Albert	[H] mix		
NJ	Englewood	Funsch, Dr. Robert	4, 43, 47, 49, 51, 60, 88, 90	1978	07631
NJ	New Brunswick	Reeser, Paul	4, 43, 47, 49, 51, 60, 88, 92, 171, 172	1978	08903
NY	Albany	Birmingham, Mike	HM2:524, 850, 852, 868, 1056+ HI2:	1983	12233
NY	Red Creek	Douglass, Earl	43	1976	13143
NY	Northville	Nielsen, Rodney R.	4, 43, 47, 49, 51, 60, 88, 92, 171, 172	1980	12134
NY	Armonk	Segretti, Ralph	Slurry 10	1983	
NY	Armonk	Segretti, Ralph	47, 50, 234, 420, 504, 518	1980	10504
NY	Armonk	Segretti, Ralph	4, 43, 47, 49, 51, 60, 88, 92, 171, 172	1981	10504
NY	Albany	Stross, Raymond G.	[I]	1984	12222
NY	Ithaca	Webb, Walt W.	43	1976	14853
NY	Selden	Welch, Ray	4, 43, 47, 60	1978	11784
OH	Wooster	Leben, Curt	43	1976	44691
OH	Columbus	Powell, C. C.	43	1976	43210
OH	Columbus	Rutter, Milton R.	4, 43, 47, 49, 51, 60, 88, 92	1979	43210
PA	Johnstown	Harris, Dr. James	6, 14, 34, 36, 44	1977	15904
PA	Pine Grove	Kohr, Clarence	Slurry of 10		17963
PA	Johnstown	Mackey, Dr. Halkar	Bar V, Bar H, 2043 H		15904
PA	Lancaster	Reed, Henry	43	1976	17602
PA	Middletown	Towers, Barry	14, 43, 49, 51, 88, 90, 92	1978	17057
TN	Knoxville	McCarroll, David	Bar H, 2043 H	1975	37901
TX	Commerce	Pullen, Arthur	4, 43, 47, 49, 51, 60, 88, 92, 171, 172	1982	75428
VA	Charlottesville	Artman, Joel	9, 14, 49, 50, 60, 61, 90, 102	1977	22903
VA	Charlottesville	Artman, Joel	4, 43, 47, 49, 51, 60, 88, 92	1978	22903
VA	Alexandria	Brill, Donald N.	43	1976	22306
VA	Arlington	Chandler, Hudson	4, 43, 47, 60	1978	22204

VA	Arlington	Chandler, Hudson	4, 43, 47, 60	1978	22204
VA	Arlington	Chandler, Hudson	4, 43, 47, 49, 51, 60, 88, 92	1979	22204
VA	Blacksburg	Griffin, Gary J.		1976	24061
VA	Bridgewater	Heisey, Dr. Lowell			22812
VA	Richmond	Matheny, Dr. W. H.	43	1976	23209
VA	Richmond	Schweitzer, D. J.	14, 53, 43	1977	23209
WI	Madison	Reeser, Paul		1982	53711
WV	Morganstown	MacDonald, William	Bar H, Bar V	1975	26505

Chestnut Germplasm available in Connecticut. Trees are in Experiment Station plantings (which include over 1000 trees) unless otherwise noted.

CAES orchards include the ortets of the following cultivars:

1. 'Sleeping Giant'
2. 'Mahogany'
3. 'Essate Jap'
4. 'Toumey'
5. 'Lockwood'
6. 'Little Giant'
7. 'Hope'
8. 'King Arthur'

and grafted trees of the following cultivars:

1. 'Clapper'
2. 'Colossal'
3. 'Eaton'
4. 'Orrin'
5. 'Lenoir'

Various important species trees in Connecticut

Japanese chestnuts *Castanea crenata*

1. three trees planted in 1876, probably ` Parsons' Japan' all on private land (First Congregational Church, Cheshire; Bee and Thistle Inn, Old Lyme; P.T. Barnum house, Bridgeport)
2. USDA-PI #78626, seed from wild trees in Oguriyama, Amori-ken, Japan, planted 1933 (orchard tree)
3. USDA-PI #104015, Nobeoka Eirinsho, Yokugomura, Higashi, Usuki-gun, Miyasaki-ken, Japan (32 deg. latitude, planted 1935 (orchard tree)
4. USDA-PI #104016, Numakunai Eirinsho, Ippoimura, Iwate-gun, Iwate-ken, Japan, planted 1935 (timber tree)
5. USDA-PI #113679, Iwate-ken, Japan, planted 1939 at the CT Arboretum in New London (orchard trees)

Seguine chestnuts *Castanea seguinii*

1. one surviving tree of USDA-PI #70317, "Mo-lut-tsz" Chiuhwashaan, Anhwei, China, planted 1929 (dwarf species) and one cross (1998)

Chinese Timber Chinquapins *Castanea Henryi*

2. one tree of USDA-PI #104058, "Chu-Lee" or "Chun Lee" "pearl chestnut," Hsiaohsing, Anhwei, China, planted 1935
3. five trees of GA 30 and two of GA 31 from seed produced at Callaway Gardens in Georgia, planted 2011

Chinese chestnuts *Castanea mollissima*

1. two trees of USDA-PI #70315, "hardy trees native to north-eastern China" planted 1929, one is Graves' 'Mahogany' (timber trees)
2. USDA-PI #78744, "Tiger Paw" from the Fa Hua Ssu Temple near Beijing, planted 1932 (orchard tree)
3. two trees of USDA-PI #104061, `Lui An' Chekiang Province, China (28-32 deg. latitude) planted 1935 (timber trees)
4. four trees of USDA-PI #104063, `Kuei Lee' "large chestnut," Hsinteng, Chekiang Province, China, planted 1935 (timber trees)
5. two trees of USDA-PI #39721, from Tientsin, China, planted 1916 at the Bartlett Arboretum in Stamford (timber trees)
6. one tree of Frank Meyer's import PI #36666, from the Pingchuan region N.E. of Beijing, planted 1917 at the Bartlett Arboretum in Stamford CT (this orchard tree is the cultivar `Bartlett')
7. grove of trees of USDA-PI #58602 from north eastern China, planted 1926 in Dayville. There are vigorously naturalizing.seedlings in surrounding fields(1992).
8. 60 trees of 'Mahogany' x 'Nanking' (WL R1T15 PI #70315 and pollen from Greg Miller, PI #108552). These are from the Fagaceae Genetics Project and were planted in 2010.

European chestnuts *Castanea sativa*

1. one tree from wild seed collected in the Black Forest in Germany, planted 1985
2. 15 trees from four areas with wild chestnut near Bursa, Turkey, planted 1991
3. 21 trees from six eastern areas along the Black Sea in Turkey planted 2008
4. one tree from seed collected by Fred Paillet in the Cavcas Biosphere Reserve in the Caucasus Mountains of southern Russia, planted 1994

American chestnuts *Castanea dentata*

1. about 250 trees, seedlings from Michigan, Wisconsin, New York, and Connecticut, kept alive by hypovirulence in the blight fungus population
2. two grafts of the tree "Scientist's Cliffs" from land of Flippo Gravett in Port Republic, Maryland; had measurable resistance to chestnut blight (see Anagnostakis, 1992) (Sandy doesn't think it is a *dentata*)
3. 8 trees on Painter Hill Road, near Painter Ridge Rd., in Roxbury, used in Experiment Station crosses almost yearly from 1948 to 1961, may have some resistance to blight

American Chinquapins

1. *pumila*, one tree from Empire Chestnut Co. planted 2000, three trees from MD planted 2014
2. *ozarkensis*, 57 trees from the Ozark Plateau in OK, planted 2004 and 2005

3. *alnifolia*, two trees from Lafayette County, FL planted 1995
4. *floridana*?, four trees from north FL planted 2011

Older hybrids of special interest
(*sativa* X *crenata*) X *dentata*
two trees planted 1931, one the "Smith hybrid" and one called "Hammond-86"
mollissima X *dentata*
four trees planted 1960
dentata X *mollissima*
two trees planted 1936
(*mollissima* X *dentata*) X *dentata*
two grafts of the `Clapper' tree and one tree from seed planted 1955, called `Graves'

A. Controlled crosses:

No controlled crosses were made in 2017 due to frequent rain during flowering season.

B. Information on the web

Some of the cards which record chestnut trees sent out by the USDA to people in the US (and elsewhere) are now scanned and on my web page, www.ct.gov/caes/sla
Those finished are Indiana, Oregon, Washington, and Massachusetts. In addition, all of my fact sheets are there, as well as the scan of the complete CAES breeding record.

Kirsten Hein, University of Tennessee, Chattanooga

Implementing early screening methods to detect resistance to *Phytophthora cinnamomi* in first-backcross Chinese-American chestnut hybrids. Phytophthora root rot (PRR), caused by the oomycete *Phytophthora cinnamomi* Rands, is one of the greatest obstacles to growth of American chestnut (*Castanea dentata* Borkh.) in large portions of the species' range. The objective of our research is to implement early screening methods for PRR resistance in Chinese-American chestnut hybrids. Developing early and reliable screening methods can facilitate the efficient introgression of PRR resistance from Chinese chestnut (*C. mollissima* Blume) into potentially blight resistant advanced hybrid populations. This study tests the efficacy of a novel method for identifying PRR-resistant hybrid chestnuts in a greenhouse setting. The hybrid families to be studied are derived from twenty years of work by the Tennessee Chapter of the American Chestnut Foundation (TACF®) and represent sources of PRR resistance not previously utilized by TACF®. Individuals were inoculated with *P. cinnamomi* prepared on V8 agar medium and a rice-grain inoculum, during the midsummer months, and later screened at the end of the growing season. The PRR symptom severity of plants was evaluated following methods previously developed at Clemson University. Survival quotients of the American-Chinese backcross families ranged from 8% to 39%, while the survival quotients of the American and Chinese controls were 20% and 58%, respectively. Higher survival quotients of several backcross families relative to the American chestnut control group suggests

that PRR resistance was inherited by a large portion of backcross progeny in these families. The 58% survival quotient observed in the Chinese chestnut control group implies that high disease pressure existed in our greenhouse screening conditions. Future work will include a second year's collection of data with different hybrid families and the application of additional statistical analyses to determine which crosses will be repeated in future breeding efforts.

Bert Abbott, University of Kentucky

TreeSnap. Abbott reported on TreeSnap, a citizen science tool used to help our forests. This program for mobile phones (Apple and Android) was produced by Abbott and the following individuals:

- Ellen Crocker, Forest Health and Education Center, University of Kentucky
- Bradford Condon, Abdullah Almsaeed and Meg Staton, Univeristy of Tennessee
- Dave Nelsen, Forest Health and Education Center, University of Kentucky/USDA-FS

Invasive diseases and pests threaten the health of America's forests. Scientists are working to understand what allows some individuals trees to survive, but they need to find healthy, resilient trees in forest to study. That is where concerned foresters, landowners and citizens can help. TreeSnap is used to tag trees found in communities, on private property or in the wild. Scientists can then use data collected to locate trees for research projects like studying the genetic diversity of tree species to aid in building better tree breeding programs.

TreeSnap is available on the Apple AppStore and Google Play. TreeSnap is easy to use. Snap a photo, answer a few questions and the data collected (GPS location, crown health, burs, flowers, blight, etc) can be sent in a short time-frame. The GPS location of each tree is protected and not revealved to outside parties. TreeSnap only shows relative locations of trees (5-mile radius). Only TreeSnap-related research programs will have access to the information.

Meg Staton's group does the bioinformatics. This program (TreeSnap.org) is an improved format for reporting surviving trees and it has the potential for new partners.

Hill Craddock, University of Tennessee, Chattanooga

The Carter Center. Craddock reported that on 19 July 2017, invited guests were honored by the presence of former President Jimmy Carter at a "Celebrating the American Chestnut" event dedicating the recently expanded Carter Center chestnut orchard. Following remarks by GA-TACF's Scientific Coordinator and Treasurer Dr. Martin Cipollini (Berry College) and TACF's President & CEO Lisa Thomson, President Carter spoke of his long-term interest in and continued commitment to the restoration of the American chestnut. As the event got underway, guests wandered freely about the orchard and engaged GA-TACF Board members and TACF® staff with a myriad of questions about the restoration of American chestnuts in Georgia and region-wide. All that buzz ended and a new one began as President Carter made his way across the lawn to join the group. This photo shows the group gathered at the end of the event with President Carter seated at the middle front row, and daughter-in-law Elizabeth Carter (Carter Center Board of Councilors) standing in red at the far left.

The Chattanooga Report. Craddock reported on 'big ugly' cankers located in Lincoln County, TN. Most have hypoviurlence in addition to increased resistance. At some of the high elevation sites (>4,000 ft), Craddock is testing Japanese chestnut along with hybrids 'Gideon', 'Payne' and 'Lindstrom'. These sites have ambrosia beetles. Once the trees are large enough, they will be inoculated with *C. parasitica*. This work (inoculation and subsequent measurements) is conducted 100% with volunteers.

In orchard tests, trees with intermediate resistance are rogued to allow only trees with the best resistance to remain. Craddock is trying to populate seed orchards and he needs volunteers to maintain the trees and manage the orchards.

Craddock has a site near Knoxville that was planted by direct seeding of nuts. He is also working on a BC_3F_3 orchard at Bendabout Farms in Bradley County, TN. One of the advantages of direct seeding is that transplanting sometimes can harbor *P. cinnamomi*.

Craddock is using graft propagation to propagate cultivars and he wants to use graft propagation to propagate rare chestnuts. Grafted trees often bloom at a young age. Craddock wants to have rootstock as closely related to scion as possible; however, he often looses American rootstock. He wants to get chestnut outside the refugium if possible.

This summer while visiting relatives in Virginia, Craddock's brother-in-law is keeping bees near the Lesesne State Forest, near an area where Dick Jaynes and Tom Dierauf planted trees decades ago. The trees are now quite large and although they were never inoculated with hypovirulent isolates, they have large, superficial cankers. Gary Griffin (Virginia Tech) has large surviving American chestnuts that have been grafted onto local rootstock.

The chestnut: a tree crop archetype. Chestnuts harvested by hand is a tradition in many groves in Italy. He showed pictures of hand harvesting chestnuts in a grove near Avellino, Italy. Many regions in Italy use grafted chestnuts and Craddock noted that grafted trees can live a long time. Craddock is working with growers to grow chestnuts as a nut crop using grazing animals rather than herbicides. Sheep are used to "mow" around chestnuts and Craddock likes this as a pasture system.

Chinese chestnuts. Craddock noted that chestnut blight on Chinese chestnuts this year is worse than he has ever seen before.

Taylor Perkins, University of Tennessee, Chattanooga

Genotyping-by-sequencing and chloroplast DNA analysis of the North American *Castanea* species and hypothesized hybrids. Plants that display a combination of morphological traits from American chestnut (*Castanea dentata*) and Allegheny chinquapin (*C. pumila*) occur in the Southern Appalachians, where the two species grow in sympatry. Throughout the 20th century, authors have hypothesized that such plants were derived by hybridization between *C. dentata* and *C. pumila*, referring to the putative hybrids as *C.* × *neglecta* and *C.* × *alabamensis*. Alternatively, other authors have argued that the purported hybrids from Alabama are actually eastern disjunct populations of Ozark chinquapin (*C. ozarkensis*). We tested the above hypotheses by analyzing DNA sequence data from the nuclear and chloroplast genomes of *C. dentata*, *C. pumila*, *C. ozarkensis*, suspected hybrids, and two outgroup species, Chinese chestnut (*C. mollissima*) and Japanese chestnut (*C. crenata*). Sanger sequencing of six noncoding chloroplast DNA (cpDNA) loci showed that *C.* × *alabamensis* accessions possessed M-haplotypes, one of which was shared with sympatric *C. dentata*. Genotyping-by-sequencing of nuclear genomic DNA from 103 accessions yielded 124,073 single-nucleotide polymorphism (SNP) loci. Phylogenetic analysis and principal components analysis (PCA) of the SNP dataset revealed that all *C.* × *alabamensis* accessions clustered with one *C. ozarkensis* accession, and this group was sister to all *C. pumila* accessions. Phylogenetic analysis and PCA placed the second *C. ozarkensis* accession between the *C. pumila* and *C. dentata* clusters. Preliminary results suggest that: (1) *C.* × *alabamensis* populations are either an eastern disjunction of *C. ozarkensis* or are derived from hybridization between *C. ozarkensis* and *C. pumila*; (2) the second *C. ozarkensis* accession may have genomic signatures of introgression from *C. dentate*; and (3) shared cpDNA haplotypes between co-occurring *C. dentata*, *C.* × *alabamensis*, and *C. pumila* may be indicative of past interspecific hybridization and chloroplast capture. Additional analyses to test for admixture in the nuclear genomes of the accessions are planned.

Rita Costa, INIAV (Instituto Nacional de Investigação Agrária e Veterinária), Oeiras, Portugal

Improvement of chestnut for resistance to *Phytophthora cinnamomi*: an overview of the breeding program and genomic approaches on course in Portugal (in conjuction with T. Zhebentyayeva, Carmen Santos, H. Machado, J. Gomes-Laranjo and C.D. Nelson). Root rot, also known by ink disease, caused by the oomycete *Phytophthora cinnamomi*, is currently the most severe disease and main threat for European chestnut (*Castanea sativa*) in Portugal and

Europe. Inter-specific controlled crosses were established between *C. sativa* x *C. crenata* and *C. sativa* x *C. mollissima* for introgression of resistance genes of the Asian species into the susceptible *C. sativa* and two mapping populations were produced. These crosses had two purposes: the first, to create mapping populations for identifying Quantitative Trait Loci (QTL), genetic factors that control resistance to *P. cinnamomi*, the second, for selecting resistant hybrid genotypes for mass propagation of improved rootstocks, for future release to the industry, which presents a serious deficit of improved plant material in Portugal and Europe. *Phytophthora cinnamomi* susceptibility of each progeny was evaluated by root and cuttings inoculation tests. For each test, biological replicates of each genotype were used. The lesion progression rate in the cuttings inoculation test was strongly and positively correlated with the survival in the root inoculation test. Though, the cuttings inoculation test proved to be a reliable and expedite method for screening large numbers of genotypes regarding resistance to *P. cinnamomi,* and a good phenotyping tool for QTL analysis. An overview of the entire breeding program and the genomic approaches in progress was presented, with the main results obtained so far.

Steve Jeffers, Clemson University

In collaboration with: Chestnut Return Farm (Seneca, SC), The American Chestnut Foundation (TACF®), and the USDA Forest Service

Overview of work:

- Molecular genetics of resistance to *Phytophthora cinnamomi*.
- *Phytophthora* spp. associated with American, Chinese and backcross hybrid chestnut seedlings planted in the southeast.
- Screeening backcross hybrids for resistance to *Phytophthora cinnamomi.*
- Distribution of *Phytophthora* spp. in soils where chesntuts are growing or will be planted.

Overview of *Phytophthora cinnamomi*. It is generally believed that *P. cinnamomi* is native to southeastern Asia and, perhaps, South Africa. It is believed that this pathogen was introduced accidently into the coastal region of the southeastern U.S. in soil or on the roots of containerized plants imported from Asia in the late 1700s; it then spread inland with the human population. During the mid-1800s, devastating losses to American chestnut and native chinkapin trees (*Castanea pumila*) in the Coastal Plain and Piedmont regions throughout the southeastern U.S. have been attributed to PRR, with widespread death of trees occurring long before chestnut blight arrived in North America. Phytophthora root rot was first reported on American chestnut in 1932 and later confirmed over the next 10-15 years.

P. cinnamomi is one of the most economically important plant pathogens worldwide. It is known to attack over 1000 host plants, and it has been speculated that several thousand more plant species are susceptible in Australia alone. *P. cinnamomi* has caused numerous destructive diseases on agricultural, ornamental, and native plants around the world: dieback of eucalyptus trees and numerous understory species in forests of western Australia; mortality of oaks in Mexico; little leaf disease of shortleaf pines and root rot of Fraser fir Christmas trees in the southeastern U.S.; heart rot of pineapple and root rot of ohia trees in Hawaii; blight and canker of macadamia trees; root rot of avocado; root rot and trunk cankers on many hardwood

and conifer trees; and root and crown rot on many different nursery and landscape plant species—particularly camellia and members of the Ericaceae (e.g., azalea, heath, Pieris, Rhododendron, etc.). *P. cinnamomi* is considered to be a fairly uniform clonal population in most regions where it occurs, with the A2 mating type dominating most local populations and phenotypic and genotypic variation relatively low. However, over the years, pathogenic variation within this species has been reported. Recently, we have identified genotypic variation that correlates with phenotypic variation in a population of *P. cinnamomi* from ornamental plants.

Diversity and pathogenicity of species of *Phytophthora* associated with hybrid American chestnut seedlings. This work was completed by M.S. graduate student, Ms. Suzette Sharpe. She completed her degree in May 2017. She investigated the diversity of *Phytophthora* spp. associated with root rot on American, Chinese, and hybrid chestnut seedlings (developed by TACF®) that were planted in test plots in forest sites in three southeastern states: NC, VA, and TN. These plots are part of a research project being conducted by Dr. Stacy Clark with the USDA Forest Service. Since 2011, over 600 samples have been received and 242 Isolates of *Phytophthora* spp. have been recovered from roots and soil. The two main objectives of this project were to: (1) Identify and characterize all isolates of *Phytophthora* spp. based on morphological, physiological, and molecular traits; and (2) determine pathogenicity of all species of *Phytophthora* associated with American chestnut using Koch's postulates. Sharpe isolated *Phytophthora* species from the following counties.

Virginia	**Species of *Phytophthora* isolates**
Augusta County	*P. cinnamomi*
Giles County	*P. cinnamomi, P. cambivora*
Wise County	*P. cinnamomi, P. quercetorum*
Tennessee	
Carter County	*P. cinnamomi, P. cambivora*
Cocke County	*P. cinnamomi, P. cambivora*
North Carolina	
Graham County	*P. cinnamomi, P. cambivora, P. heveae*
Clay County	*P. cinnamomi*
South Carolina	
Oconee County	*P. cinnamomi, P. cryptogea*

Results from this project have been very interesting and enlightening. *Phytophthora cinnamomi* is reported to have been active in the U.S. southeast as early as 1824. For over 80 years, only *P. cinnamomi* has been associated with American chestnut. However, *P. cinnamomi* (primarily), *P. cambivora*, *P. heveae* (infrequently), have been recovered and several isolates of *Phytophthora* spp. have yet to be identified. Sequencing of rDNA-ITS and several other genes is being conducted to verify identities of these species. We have determined the mating type of all heterothallic isolates and screened all isolates for sensitivity to the fungicide mefenoxam. In pathogenicity tests on American chestnut seedlings, *P. cinnamomi* was most aggressive—as expected—but the other species of *Phytophthora* also were capable of colonizing roots and causing disease. We are in the process of completing this project, but it appears that there are

species of *Phytophthora* other than *P. cinnamomi* that are capable of causing Phytophthora root rot on American chestnut.

In summary, five species of *Phytophthora* have been recovered form plant and soil samples. *P. cinnamomi* was recovered most frequently while *P. cambivora* was recovered from six field sites and a nursery in VA. *P. heveae* was recovered from one site in NC, *P. quercetorum* from one sample in VA and *P. cryptogea* from one field in SC.

Two greenhouse trials have been conducted to test pathogenicity. Soil in tubs was infested with one isolate of each species of *Phytophthora*. Five American and five Chinese chestnuts were inoculated with each Phytophthora species; the seedlings were 2-3 years old. The tubs were flooded every 48 h for two weeks. Data is as follows.

	American Chestnut		Chinese chestnut	
	Tap roots with lesions	Pathogenic isolate	Tap roots with lesions	Pathogenic isolate
P. cinnamomi	10 of 10	10 of 10	0 of 10	0 of 10
P. crytogea	7 of 10	10 of 10	4 of 10	7 of 10
P. cambivora- VA 2	5 of 10	7 of 10	1 of 10	10 of 10
P. cambivora- VA 1	3 of 10	5 of 10	2 of 10	5 of 10
P. cambivora- TN 1	5 of 10	6 of 10	0 of 10	1 of 10
P. cambivora- TN 2	4 of 10	5 of 10	0 of 10	5 of 10
P. cambivora- NC	2 of 10	8 of 10	0 of 10	2 of 10
P. heveae	2 of 10	9 of 10	0 of 10	5 of 10

Summary of pathogenicity test. All species caused necrotic lesions on the main tap root of American chestnut. Significant root rot was caused by three species: *P. cinnamomi, P. cambivora* and *P. cryptogea*. *P. cinnamomi* and *P. cambivora* killed some American chestnuts. Disease severity was greatest with *P. cinnamomi*.

Virulence of *P. cinnamomi* and *P. cambivora* was compared in greenhouse tests. There were 10 treatments (control, 5 treatments with *P. cinnamomi* and 4 with *P. cambivora*). *P. cinnamomi* was more virulent than *P. cambivora* and there was no difference in virulence among isolates of each species.

Backcross screening for PRR resistance. Screening backcross trees for resistance to *P. cinnamomi* was conducted in cooperation with TACF® and the Joe James Chestnut Returm Farm, Seneca, SC. This year (2017) was the 14th year of continuous testing; testing began in 2004. Twenty BC_3F_3 families were evaluated along with American and Chinese chestnuts as controls. The basic protocol is as follows. Germinated seeds of American, Chinese, and hybrid chestnut are planted outside in April in 570-L plastic tubs filled with a soilless container mix (Fafard 3B). The tubs are infested 12 to 14 weeks after planting with a mixture of two isolates of *P. cinnamomi* originally recovered from diseased chestnut seedlings growing at the study site. After infestation, the container mix in each tub is brought to saturation at least once while plants are actively growing to encourage disease development. Plants begin to die within 2-3 weeks. In December, each seedling is uprooted and scored for mortality and PRR severity (using a standard 0-3 scale) by visually examining the roots and lower stem.

In these tests, *C. dentata* seedlings consistently have been susceptible and died, and *C. mollissima* seedlings consistently have been resistant and survived. From 2004-2015, over 200

hybrid families have been tested from generations that ranged from F_1 to BC_4. The 2017 test is in progress. Families with seedlings resistant to *P. cinnamomi* have occurred each year, but the number of resistant seedlings and PRR severity ratings varied considerably among families—depending on which families were being screened.

Jeffers continues to collaborate Dr. Tatyana Zhebentyayeva here at Clemson, Paul Sisco and Jared Westbrook at TACF®, Bert Abbott's research group at the University of Kentucky to identify genes associated with resistance to *P. cinnamom*i.

Distribution of Phytophthora spp. in chestnut soils. In collaboration with TACF®, soils have been assayed for *Phytophthora* spp. at Clemson University. They have assayed soils where chestnut is growing or where it might be planted. This assay has been ongoing for 10 years. Jeffers has hundreds of isolates of *Phytophthora* spp saved in a permanent culture collection. Jeffers has samples from 10 states (AL, GA, KY, MD, MO, NC, PA, SC, TN and WV). The following table lists the number of soil samples that are positive for *Phytophthora* spp.

Year	# Soil Samples	# Positive for *Phytophthora*	% Positive
2015	53	16	30%
2016	17	6	35%
2017	45	12	27%

Business Meeting

Administrative advisor, Bradley Hillman was unable to attend. NE-1333 is entering the last year of the current 5-year project (Oct 2013-Sept 2018). There was consensus that the project should continue as the structure offered by a regional project allows for good interaction among participants. Fred Hebard (TACF®, emeritus) agreed to spearhead the writing of a new proposal. Hebard would like everyone's input, under the three objectives, submitted to him by 1 November 2017. This way, Hebard will have time to write the proposal and have it submitted to Administrative Advisor Hillman by the end of November 2017. The termination date of the project is 30 Sep 2018, so the 2018 meeting must be held prior to 01 October.

There was a Friday afternoon field trip to the Bent Creek Experiment station to look at small stem assays of backcross seedlings and Phytophthora assays in the greenhouse. We also visited the Pryor seed orchard in Edneyville, NC where Paul Sisco provided a tour.

The meeting adjourned at 4:00 pm followed by an optional hike to Hemphill Bald.

John Carlson is the chair-elect and he agreed to host the meeting at State College in 2018. The meeting date has already been set for 2018. It will be held 7-9 Sept 2018 at the Nittany Lion Inn in State College. Carlson stated that we can visit the TACF® BC_3F_2 trials set up by Sara Fitzsimmons, and Kim Steiner can offer a tour of the Penn State arboretum.

Carlson announced that the Schatz Center may be able to offer some financial support for the meeting.

<u>Possible Schatz Center contributions</u>:

- Student, post-doc, junior faculty travel awards
- Reception
- Keynote talk(s) from guest speaker(s)
- Project/proposal planning session?

- Workshop opportunities
    - Genome website
    - Genome-wide selection demonstrations

Respectfully submitted,
Mark Double
West Virginia University
October 2017

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Milestone Accomplishments

- A thorough analysis of the four *C. parasitica* RNA-dependent RNA polymerase (*rdr*) genes was completed during this reporting period. Disruption mutants were made for each of the *rdr* genes independently. Double (*Δrdr1/Δrdr3* and *Δrdr2/Δrdr3*) and triple *Δrdr1/Δrdr2/Δrdr3* mutants were also made to overcome potential problems of functional redundancy (a quadruple mutant was not prepared because *rdr4* appears to be a pseudo-gene). None of the *rdr* disruption mutants displayed any growth or morphology phenotypes that differed from the wild-type strain either with or without hypovirus infection. Deletion of the *rdr* genes also failed to result in detectable changes in transposon expression or hypovirus recombination activity. We conclude that *rdr* genes in *C. parasitica* do not have significant roles in RNA silencing as part of defense responses against mycoviruses or transposons or have a significant role in viral RNA recombination has we have shown previously for *dcl2* and *agl2*.
- A simple and efficient system was developed by adapting the Cre-*loxP* recombination system for unlimited recycling of the limited number of available selectable marker genes (SMGs). The successful application of this method to *Metarhizium robertsii* suggests potential use for optimizing reverse-genetics analysis in a broad range of filamentous fungi.
- Mutational analyses of the infectious CHV-1/EP713 infectious cDNA clone defined the requirements for autocatalytic cleavage of papain-like leader proteases p29 and p48 and the functional importance of autoproteolysis in the context of hypovirus replication. The studies also exposed an alternative p48 processing pathway independent of the encoded papain-like protease activities.
- In order to effectively determine the vegetative incompatibility genetic structure of *C. parasitica* field populations, we designed PCR primer sets that selectively amplify and distinguish alleles for each of the six known diallelic *C. parasitica vic* genetic loci. PCR assay results were validated using a panel of 64 European tester strains with genetically determined *vic* genotypes. Analysis of 116 *C. parasitica* isolates collected from five locations in the eastern United States revealed 39 unique *vic* genotypes and generally good agreement between PCR and tester strain coculturing assays in terms of *vic* diversity and genotyping. The availability of molecular tools for rapid and precise *vic* genotyping significantly improves the ability to predict and evaluate the efficacy of hypovirulence and related management strategies.
- The identification of vic genes and adaptation of the Cre-*loxP* recombination system in previous years allowed us to systematicly disrupt multilocus *vic* genes and excize exogenic genes to generate strains of the chestnut blight fungus able to transmit hypovirulence to strains with genotypic differences at any or all of the defined *vic* loci. The results demonstrate the feasibility of modulating fungal allorecognition to promote transmission of virulence-attenuating mycoviruese for enhanced biocontrol potential. These “Super Donor” strains are currently being tested in USDA permitted field trials near Grantsville, MD. Results from a 1-year study involving naturally occurring cankers challenged with

super donor strains indicate significantly less canker expansion compared to cytoplasmic hypovirulent isolates (without gene knockouts) and water agar controls.

- A BC_3F_3 orchard was established at the Univeristy Forest in Preston County, WV as a demonstration orchard with public access.
- Over 60,000 American chestnut backcross trees have been planted in TACF's Meadowview seed orchards since 2002. After inoculation and culling, 5000 trees remain from which to make the final selections of 500 of the most disease resistant trees.
- Version 1.1 of the Chinese chestnut genome is available to the public at the website created and curated by Dr. Margaret Staton at the University of Tennessee-Knoxville. The version 1.1 genome assembly (for TACF cv. Vanuxem) consists of 724.4 Mb in 41,270 scaffolds, averaging app. 40,000 bp in length. A total of 36,146 gene models and 38,146 peptide sequences were predicted in the genome. BAC contigs spanning the 3 blight resistance QTL (identified in the early F2 QTL mapping population) were also sequenced and assembled into 395 scaffolds.
- Leaves from SUNY-ESF's transgenic trees were used as a foodsource for tadpoles to discern if TG leaves were deleterious to leg development. For all leaf sources (chestnut and non-chestnut), In fact, tadpoles developed fastest on American chestnut leaves, both transgenic and non-transgenic.