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

Hungry Mother State Park, Marion, VA

September 10-12, 2015

Attendance:

Connecticut:	Sandra Anagnostakis (Connecticut Agricultural Experiment Station)
Kentucky:	Lynne Rieske Kinney, Anna Conrad (University of Kentucky), Tyler Dreaden (USFS-Lexington)
Maryland:	Donald Nuss, Dongxiu Zhang (University of Maryland Institute of Bioscience and Biotechnology Research, Shady Grove)
Michigan:	Andrew Jarosz, Josh Spring, Matt Kolp (Michigan State University)
Mississippi:	Dana Nelson (Southern Institute of Forest Genetics, Saucier)
New Mexico:	Angus Dawe (New Mexico State University)
New York:	Kristen Stewart-Russell, Linda McGuigan (SUNY-ESF); Steve Jakobi (Alfred Station)
Ohio:	Leila Pinchot (USFS, Delaware)
North Carolina:	Jared Westbrook, Paul Sisco, Lisa Thomson, Tom Saielli (TACF®, Asheville)
Pennsylvania:	Gary Micsky (Penn State Extension, Mercer), Mike Marshall (Shippensburg University)
Portugal:	Rita Costa (Instituto Nacional de Investigação Agrária e Veterinária)
South Carolina:	Steve Jeffers, Tatyana Zhebentyayeva (Clemson University)
Tennessee:	Hill Craddock, Taylor Perkins, Conrad Blunck (UT Chattanooga)
Virginia:	Fred Hebard, Laura Georgi (TACF®, Meadowview), Matt Brinckman (TACF®-Charlottesville), Laurel Rodgers (Shenandoah University)
West Virginia:	William MacDonald, Mark Double, Cameron Stauder (West Virginia University)

The meeting was called to order by Chairman Hebard at 8:30 am on 11 Sept 2015 at the Hemlock Conference Center at Hungry Mother State Park in Marion, VA. John Stone, business manager of the park provided a welcome address. Jim Young, assistant manager of the park, indicated that the park consists of 2800 acres. The park is one of seven original Civilian Conservation Corps efforts in Virginia. The stone work in the park dates to 1935-1940. It is possible to see Mount Rogers, VA and areas in North Carolina from Molly's Knob, the highest point in the park.

Lisa Thomson, President and CEO, The American Chestnut Foundation

Thomson congratulated Fred Hebard on his 26 years of service to The American Chestnut Foundation and she lauded Hebard's efforts on behalf of the organization. Thomson relayed that she is not a scientist; she has degrees in biology and art but she has been in conservation for more than 30 years with a brief stint in higher education. Her passion is raising resources for something she really loves.

Thomson indicated that she is here to support researchers involved in NE-1333. She stated that there is a lot of tenure among those in attendance at the meeting and she would like to work collaboratively with members of the group. Having lived in Florida for 45 years (working on land management and fundraising for the Nature Conservancy), Thomson indicated that she knew nothing of the chestnut story and she was disturbed that the message has not touched more people. She wants to tell the chestnut story in a bigger way with the help of many people, NE-1333 members included. She is streamlining the board processes and she is looking for more involvement from people and encouraged members to join a TACF committee. The science leadership team is comprised of Sara Fitzsimmons, Jeff Donahue and Jared Westbrook, along with the regional science coordinators. The science oversight committee is chaired by Brian McCarthy (Ohio University). There is also a science advisory committee. Westbrook is working hard to update the Burnham plan that builds on Hebard's work. The current plan expired in 2014 (written in 2004). That work will be presented at the annual TACF board meeting in October at Penn State University.

Thomson has only been on board for seven months. She is strengthening ties with the US Forest Service; there was a MOU that was signed in 2010 and it will expire in 2015. She is meeting with the chief forester of NRCS in Washington, DC and associate chief Mary Wagner. This was all formulated back in April by people in Region 8 in Atlanta. Rex Mann, long-time TACF board member, was partly responsible for the USFS meeting. She commented on the strong partnerships with state departments of forestry, along with academic partners like Scott Merkle at the University of Georgia and Bill Powell at SUNY-ESF. Thomson indicated that she is working on increasing visibility of the American chestnut. Working with a single species organization is often difficult to capture peoples' enthusiasm. She has been researching best practices of other organizations (like the long-leaf pine alliance). No one does what TACF does, saving a species on the edge of extinction. She is also attempting to increase visibility through social media. The current TACF webpage is being updated (it will be unveiled in January) to make it easier for people to donate and find videos.

Chuck Leavell, a member of the Allman Brothers band and a long-time touring member of the Rolling Stones (also recorded with Eric Clapton, George Harrison and John Mayer), is a tree farmer and a chestnut enthusiast. He and his wife own a tree farm that they inherited. Thomson planted a few chestnuts on his plantation and she took along a few videographers and Leavell espoused how much he loves chestnuts. On December 1, Thomsom encouraged everyone to view the TACF website as they are embarking on a campaign to get 25,000 views in 25 days. She is hopeful that the goal can be achieved.

Thomson's expertise is in major gift and planned giving fundraising. She enjoys working with philanthropists and she hopes that their goals can be aligned with TACF's goals. She hopes to raise more money for the organization.

In the spring, she challenged the TACF board in April to look ahead. Within the strategic process she wants to define our mission and vision and guiding principles. She is thrilled that new board member, Dr. Penny Furth, a long-time NSF member in northern VA will help with the strategic planning process—something she did at NSF. Furth has convened a task force including Kathy Mays and Dick Will. They are compiling data that they will present at the October TACF annual board meeting.

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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***

Linda McGuigan and Kristen Stewart-Russell, SUNY-ESF

For review by the EPA, USDA and FDA, 'Darling 58' was submitted to be their lead event with 'Darling 54' as the back-up event. Both of these events contain the oxalate oxidase (OxO) gene but unlike some of their older events such as 'Darling 215' and 'Darling 311', they do not include the *gfp* and *ppt* genes. Several tests that were performed on the older events, such as mycorrhizal colonization of the roots (D'Amico *et al.* 2015*),* are being repeated with these new events. This is to ensure similar results to the older lines, in which no differences to wild type American chestnut trees were seen. They have been actively engaged with the regulators to determine what they need for a review. Their goal is to have enough data collected to begin the regulatory review possibly before the end of this year, but if not, within the next year.

Thirty other "candidate" genes that might be added to bolster the blight resistance or to enhance Phytophthora resistance are being examined. Twenty-seven of these genes come from Chinese chestnut while three come from additional plants. Several studies have been initiated to look at the differences between regulated and constitutive gene expression of the OxO gene. The two American chestnut cell lines used in these experiments were Ellis #1 and Zoar, both from New York trees. The vector constructs used contained either an OxO gene with a constitutive promoter, an OxO gene with a wound inducible promoter (Win3.12), or an OxO gene with a vascular promoter (VspB). Co-transformations to incorporate both the Win/OxO and the VspB/OxO were completed. Currently, the Zoar events and Ellis #1 events that were co-transformed are being tested to confirm gene presence, copy number, and expression levels. The Ellis #1 events that were transformed with only Win/OxO have been confirmed to carry the transgenes and now plants from these lines are being regenerated.

Two new American chestnut trees from New York State as well as a hybrid chestnut were established in tissue culture. Thomas Deacon, a TACF-NY member, provided stem cuttings of American chestnuts from stump sprouts at the bases of what were once majestic timber-type trees. The dormant stems were forced to flush new growth, sterilized, and placed on American chestnut growth medium. The two lines, named 'Deacon #2' and 'Deacon #5', are well established in sterile culture and are being multiplied for rooting experiments. 'Colossal' hybrid chestnut, provided as grafts by Jeff Zarnowski of Z's Nutty Ridge LLC, also has been established in tissue culture. The shoots currently are being multiplied and rooted.

The SUNY-ESF research team has produced over 21,000 rooted chestnut shoots *in vitro* between April 2014 and April 2015. Roughly one-third (7,860) of these were produced between January and April of 2015. With the current method, it is necessary to produce such a large number of rooted shoots to get 200 to 300 trees into the field. A number of studies to improve the efficiency are being carried out. One enhancement may be a transition from *in vitro* rooting

to *ex vitro* rooting. In collaboration with Christie-Anne Lovat, a PhD candidate from McGill University in Quebec, the quality of the *ex vitro* rooting process has been increased. With her help, rooting and survival rates have been boosted for their tissue culture plantlets. Additionally, work has been conducted to enhance plant health and growth rates in greenhouses. They anticipate this will ultimately translate to improved survival in the field.

Fieldwork has included planting and pollinating. Close to 250 new transgenic trees in Syracuse, NY were planted and 12 transgenic trees at a new "simulated restoration" site in Westchester County, NY. Also, approximately 100 non-transgenic Mother Trees at three different locations around the Syracuse area were planted. Controlled pollinations were done with wild-type trees and transgenic pollen inside their USDA APHIS permitted plots. These crosses should confirm that the transgenes are inheritable as expected; they already have seen this with previous transgenic events. The resulting nuts will be tested for nutritional content.

Disease resistance testing continues for transgenic American chestnut cell lines containing cis- and transgenes. Leaf inoculation assays have been used to screen events with several putative resistance genes from Chinese chestnut, as well as one from grape. Field-collected leaves were infected with *Cryphonectria parasitica* strain SG2-3. After an incubation period of 4 to 7 days, the area of necrosis was measured and compared to both American and Chinese ('Qing') chestnut leaves. To date, the most promising cisgene encodes a predicted acid phosphatase that was cloned from Chinese chestnut; enhanced blight resistance in three separate transgenic events containing this gene has been observed. They have six other cisgenes that have appeared to enhance blight resistance in preliminary tests, but these need to be examined further. The transgene encoding the detoxifying enzyme Oxalate Oxidase has continued to show promise with effective blight resistance and negligible negative effects. Because this gene construct looks so good in leaf and small-stem assays, they are pursuing deregulation with 'Darling 58', an event containing this gene. They recently completed a blight inoculation assay on small stems in the greenhouse, in which 6 individuals each of American chestnut, 'Darling 54' and 'Qing' Chinese chestnut were inoculated with *C. parasitica* strain EP155. After 30 days, all of the susceptible American chestnut controls had wilted completely and after 41 days, 5 of the 6 Chinese chestnut controls had wilted. All six of the transgenic American chestnuts remained healthy at the time of this report (48 days post inoculation), with healing cankers and no sign of wilt.

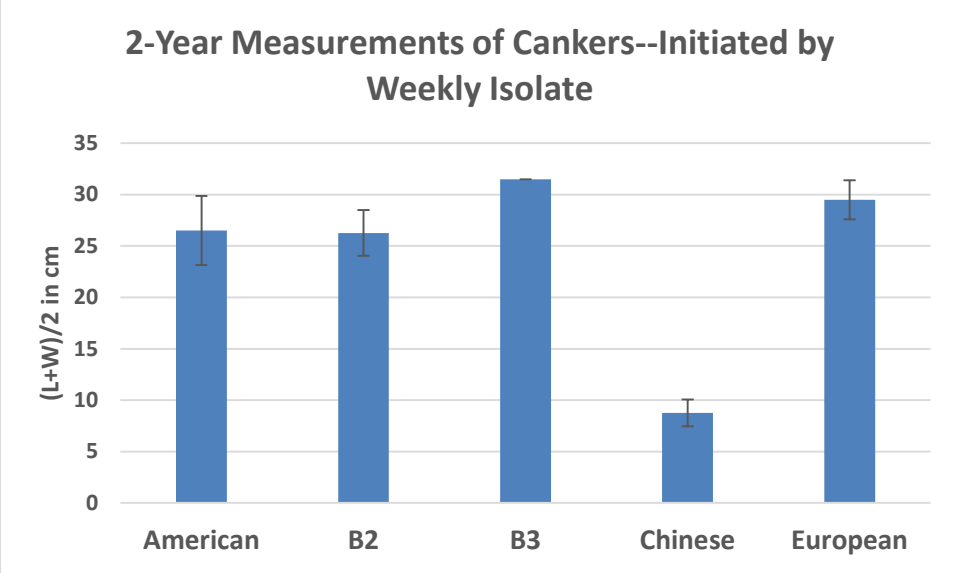
Mark Double, West Virginia University

Backcross orchard for assessment of host resistance combined with hypovirulence (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*. In three plots, naturally occurring cankers were treated with hypovirulent isolates; three plots were not inoculated. Seeds were planted annually from 2006-2011. As of July 2015, overall survival was 70%. Average diameter, height and survival data are listed in the following table.

				Average	

Species	Total	Percent Living	Percent Dead Since 2013 Inoculation	Diam. (cm)	Ht. (m)	Tallest (m)
American	181	61%	5%	3.5	3.4	9.1
B2F2	82	88%	5%	5.2	4.7	11.0
B2F3	160	66%	5%	3.4	3.2	7.4
B3F2	134	58%	3%	3.4	4.9	9.4
Chinese	189	93%	1%	5.5	5.1	10.0
European	154	42%	17%	2.6	2.5	6.8

On 31 July 2013, eighty-seven trees >3 cm (17 American; 42 BF2; 11 BF3; 25 Chinese; and 13 European) were inoculated with Weekly-2, a moderately virulent strain. Growth, sporulation and canker morphology will be assessed annually to determine host response to the inoculation with the virulent strain. Canker size [(L+W)/2] was measured in Aug 2015 two years after inoculation. The percentage of trees that have died from either artificial inoculation with WK-2 or from natural infections also was assessed in 2015, and is listed above.



All naturally-occurring cankers in the three hypovirus-introduction plots were treated during the 2013-2015 growing seasons with a hypovirulent slurry (Euro 7, COLI, GH2 and Weekly/Ep155/pXHE7). In August 2014, naturally-occurring cankers that had been treated were sampled (4 plugs/canker). Sixty-five percent (15/23) of the cankers yielded at least one hypovirulent isolate. The treated cankers will be sampled and subjectively rated annually to assess growth, sporulation and host response.

A 0-5 subjective scale was used to assess tree health (5=main stem healthy with no epicormics shoots; 4=main stem healthy with epicormics shoots; 3=main stem alive with some dieback; 2=main stem alive but badly blighted with dieback; 1=main stem dead with epicormics shoots; 0-main stem dead and no living epicormics shoots). The hv-treated and non-hv-treated plots were averaged based on species/hybrids. Ratings from an August 2015 assessment are listed in the following table.

Species/Hybrids	Tree Rating (0-5)	
	HV Plots	Non-Hv Plots
American	2.9	2.5
B2F2	4.2	3.7
B2F3	3.6	2.9
B3F2	3.2	2.4
Chinese	4.3	3.8
European	2.2	1.7

Laura Georgi, The American Chestnut Foundation, Meadowview

Molecular markers at Meadowview-Bulk sequence analysis (with Tatyana Zhebentyayeva, Dana Nelson, Fred Hebard and Albert Abbott). Almost two years ago, Georgi obtained Illumina sequences of bulks of trees from the 'Mahogany' F_2 mapping population (12 resistant and 14 susceptible). This was whole genome sequencing. The trees were pooled together, within susceptible or resistant bulks. The reads were aligned to blight resistance QTL sequences (v1.0). They obtained a number of SNPs that appeared to have significant association with one or the other bulks. Since last year, Georgi did more winnowing and found a number of SNPS that affect coding sequence and potentially the protein coding sequence. She highlighted two SNPS in close proximity to one another. She initially tested the SNPS using CAPS (cleaved amplified polymorphic sequences) and dCAPs (d=derived) makers. The cleaving is done with a restriction endonuclease, to cleave one allelic variant and not the other. dCAPs make changes in the base sequence. These were tested on 6 resistant and 8 susceptible F_2 trees that had been in the sequenced bulks. After that, Georgi identified three SNPs and conducted further testing and two of them seemed to be associated with one or the other bulks.

Trees that she used for further testing:

- 20 resistant and 23 susceptible B_3F_2 trees
- Progeny of controlled cross
- Planted in 2012, inoculated with SG2-3 in 2014

This spring she collected twigs and buds. Using CAPS104, the 'Mahogany' allele was not present in one of the parents. As a consequence, there is a backcross configuration of this marker. This did not result in significant association of the 'Mahogany' allele with resistance, possibly because of the backcross configuration. Her surprise was in dCPAS304 where there was a significant association with blight resistance and the 'Mahogany' allele.

Next steps:

- Further analysis of these data
- Would not hurt to look at these trees with more of the segregating SNPs
- Could try to improve the specificity of amplification of the SNP targeted by dCAPS229

Other work in progress:

- SSR genotyping the expanded F_2 population (with SIFG)

- Seedlings from cryopreserved pollen in conjunction with the Cincinnati Zoo (Center of Conservation and Research of Endangered Wildlife).

Marker-assisted selection will enhance the breeding program but translating markers into use in a breeding program has been a challenge. Getting assays for the markers that are cheap and reliable is not an easy task.

Potential applications for molecular markers include:

- Evaluation of genetic diversity and for the long-term restoration of the species
- Identification of cultivars, species and hybrids (there are lot of open pollinated trees; there is also the question of pollen contamination so being able to identify the father is useful.
    - Germplasm curation
    - Intellectual property protection
- Parentage analysis
- Marker-assisted selection
    - Eliminating alleles for susceptibility
- Discrimination of homozygoes from heterozygotes

Published genetic maps for chestnut blight-resistance (Cbr) quantitate trail loci (QTL)

- Kubisiak *et al.* 1997
- 101 F_2 individuals
- 184 markers, mostly RAPD
- 12 linkage groups totaling 530.1 cM (estimated >75% coverage)
- 3 Cbr QTL explaining 42/4% of variation

Published genetic maps for blight resistance QTL

- Kubisiak *et al*. 2013
    - Same population (89 individuals)
    - 520 markers, mostly Single Nucleotide Polymorphisms
    - 12 linkage groups totaling 685.7 cM
    - QTL on same three linkage groups, <10 cM intervals

The 5K Infinium Array

- Selected SNPs from earlier Golden Gate Arrays
    - 1536-SNP Chinese chestnut array
    - 768-SNP American chestnut array
- Additional SNPs to make 5K total

There was a computer error on the Golden Gate array, but still that is a lot of genotypic data available, including that from expanded F_2 population, B_3 families from Pennsylvania and a very small PRR mapping population.

Updated data from the expanded F_2 map

- 180 individuals
- 480 markers (regression algorithm)
- 12 linkage groups (plus two small one with distorted markers)
- QTL on LG B and G, possibly E and H but not F

Outcross detection using SNP data

- For markers segregating properly 1:1 AA:BB, count occurrence of the 'wrong' (BB) homozygote
- Identify trees with more than their share of 'wrong' calls

Bulk Sequences

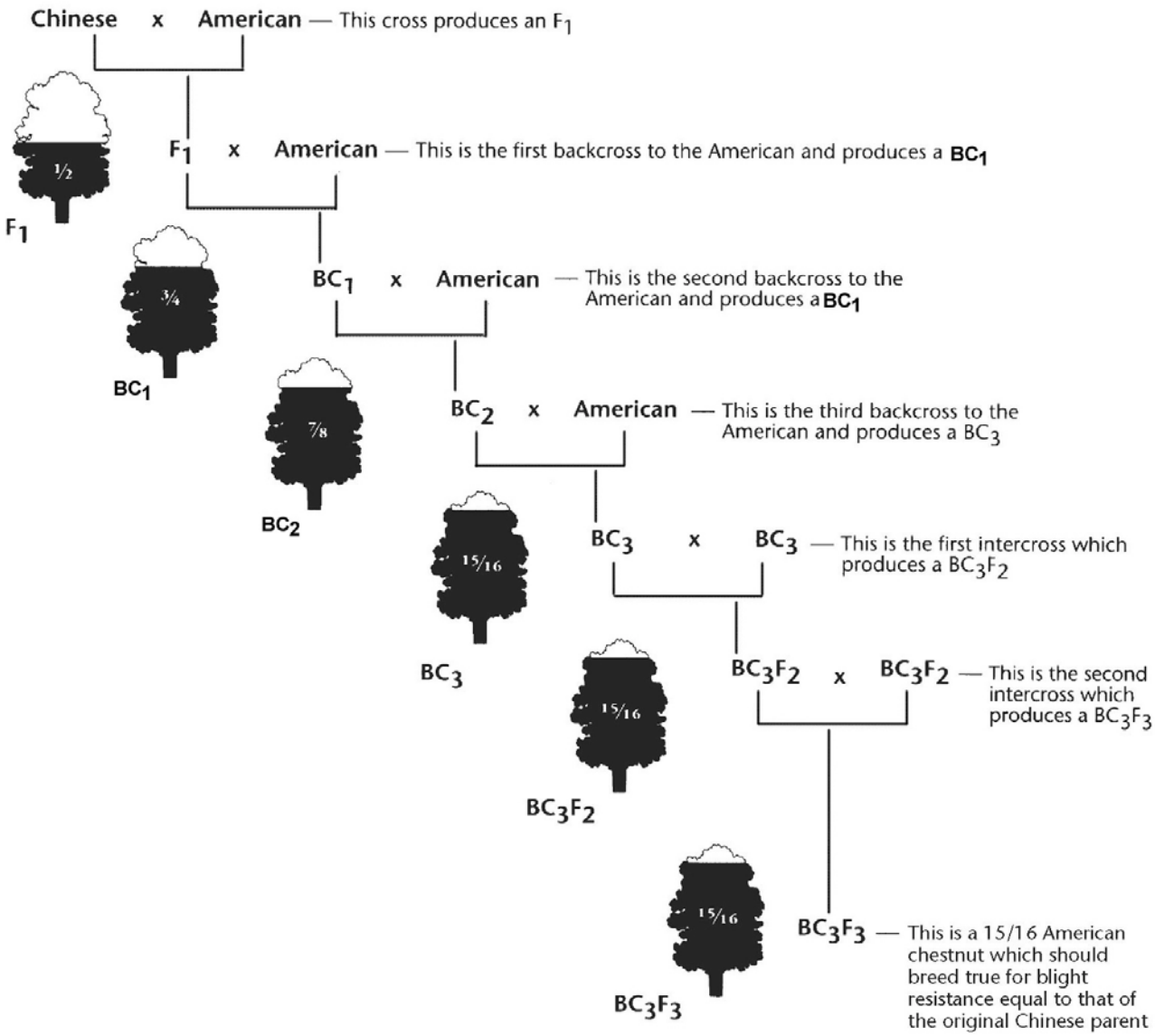
- Reads (10-15X coverage) aligned to Blight resistance QTL Sequences (v1.0) using CLC Genomics, producing Sequence Alignment/Map (SAM) files
- Allele counts generated using Galaxy tools
- G statistics calculated using JMP
- Significant SNPs evaluated for likely impact on function using hardwoodgenomics.org browser

Other work in progress

- SSR genotyping the expanded F_2 population
- Screened SSRs for genotyping a Nanking backcross population

Jared Westbrook, The American Chestnut Foundation, Asheville

Westbrook started with TACF in January as a geneticist. He presented the success of the breeding program of introgressing blight resistance from Chinese chestnut into American chestnut. The data analyses he presented was from data collected by Hebard, Georgi and others of the last 30 years. He presented the following scheme used for backcross breeding for blight resistance. This was based on the hypothesis that a few genes from Chinese chestnut control blight resistance.



Backcross breeding uses Chinese X American F_1 hybrids backcrossed to American over three generations to create hybrids that are essentially American chestnut in their genomic composition but contain blight resistance (94% American). Breeding at each generation selects for American form and blight resistance. There was an intercross among third backcross (B_3F_2s) to create trees that are homozygous for resistance at up to three loci. These trees were intercrossed (known female parent and unknown male parent, presumed to be another B_3F_2) and the seeds were planted at the Wagner and Duncan farms in Meadowview. B_3F_3s were produced.

'Clapper' and 'Graves' are both first backcross trees. The 'Clapper' tree, from Illinois, is now dead, while the 'Graves' tree (from Connecticut) is still alive. Backcross breeding lines in 'Graves' and 'Clapper' were crossed with 20 or more lines of American chestnut to create second backcross lines. About 75-100 second backcross trees generated from each of these crosses were inoculated with *C. parasitica* and then rogued out leaving 3-5 of the most resistant trees. Those were then crossed again with 20 or so American chestnuts to produce third

backcross trees. Those were rogued and tested and those were allowed to pollinate to produce B_3F_2s.

Selection in the B_3F_2 seed orchard:

- 2 sources times 20-30 lines x 9 reps per line x 150 trees per rep = 60,000 trees planted in the Meadowview seed orchards
- Aim to select 1-2 of the most blight or PRR resistant individuals per rep = 500-1000 trees
- Selected trees will be a seed source for restoration or recurrent selection.

Westbrook explained the expected segregation of blight resistance under a 2-locus model. About 75-90% of the trees will have significant canker expansion after artificial inoculation at age 3 and they will be culled from the seed orchard. That will leave 15-50 trees per plot. They still have a way to go to sift through selections. The goal is to get down to one tree per plot.

How will they sift through the remaining trees to get to the most resistant tree in each plot? A B_3F_2 parent's genetic resistance to blight is evaluated from the average canker size of its open pollinated B_3F_3 progeny relative to other B_3F_2 parents.

Trees are inoculated with two strains of *C. parasitica*, SG (low virulence) and EP155 (high virulence), strains that are at the tails of pathogenicity. There are two measures, a 1-3 canker rating (where 1=small, confined to initial lesion; 2=medium, expanded beyond initial lesion; 3=large, sunken canker and sporulating) and canker length. The two measures are combined to produce a canker severity rating.

The scope of phenotypic selection and progeny testing for blight resistance of B_3F_2 seed orchards in Meadowview are as follows:

	'Clapper'	'Graves'
Number B_3F_2 planted 2002-2014	40,000	24,000
Number B_3F_2 living after phenotypic selection	5,500	7,500
Number B_3F_2 parents progeny tested 2009-2016	384	204
Number B_3F_2 to select	300	300

There is a huge gap between the number of trees alive (ie 5,500 in 'Clapper') versus the number of trees that were tested (384). They have to figure out how to close that gap.

Canker size phenotypes of B_3F_3s span the range of Chinese and American chestnut. The B_3F_3s more closely resemble American when inoculated with Ep155 but more closely resemble Chinese chestnut when inoculated with SG. Phenotypically, there is a full range of variation in canker size among the B_3F_3s after inoculation (in the third growing season).

In an analysis of heritability of the trees that survived (after a lot of roughing), there is genetic variation (10-50% of the variation). Of the 180 'Clapper' trees (progeny tested from 2011-2014), all are significantly different greater than 0 heritabilites (going up to 50%). For 'Graves', there is only data from 85 trees but there is some significant heritability for those phenotypes (narrow sense). If you combine the Chinese+Clapper+Graves+American in a single analysis, the heritability jumps from 50-80%. In summary, progeny tests from trees with small cankers indicated that there is still significant variation in canker size. The progeny tests may

show which assumptions are correct and which are not. Progeny tests will give a more accurate measure of their genetic resistance.

Canker size breeding values for B_3F_2 trees are intermediate between Chinese chestnut and American chestnut. The lowest 5% of SG canker severity breeding values vary from 13 to 35 (closer to Chinese than American). The lowest 5% of Ep155 canker severity breeding values vary from 35 to 62 (intermediate between Chinese and American). The Chinese chestnut controls in the experiment were from Greg Miller's orchard. The American chestnuts were local. Westbrook indicated that as trees are rogued (there are still trees with alleles for susceptibility), there should be a shift of the mean of the entire population. How much it will shift is unknown, but he expects resistance to increase.

Rouging of trees in the orchard is conducted in stages. First, the trees that are deemed unacceptable based on artificial inoculation are rogued. Next, trees that have severe natural infections are rogued. Generally, there are 5-10 per plot that are left that are difficult to discern based on phenotype.

Canker severity of strains SG and Ep155 is genetically correlated. For these strains, common genes control resistance to both. Resistance seems to be common to both strains of *C. parasitica.*

Is there genetic variation in blight resistance among selected B_2 and B_3 trees? The idea at the backcross stage, once you phenotypically select for blight resistance, all the trees should be heterozygous at all loci so there should be no variation at B_2 and B_3. Based on 13,000 measurements of cankers from different American lines, there is variation in resistance at the B_3F_2 level.

Westbrook would like to sequence all of the genes in all of the backcross lines and compare gene sequences back to 'Clapper' or 'Graves' or 'Mahogany' to see what specific genes were inherited from Chinese chestnut. The second goal would be genomic selection may enable TACF to finish selection for both blight and PRR resistance in 'Clapper' and 'Graves' B_3F_2 seed orchards within five years.

Conclusions:

- The observation that some B_3F_2 trees are highly resistant to blight and also have a ~ 94% American chestnut genetic background suggests that major genes for blight resistance have been retained.
- Average blight resistance at B_3F_3 is expected to increase after selections at B_3-F_2 are complete.
- Variation at B_3 suggests that resistance alleles have been differentially lost among backcross lines.
- Genomic selection may accelerate selection and guide crosses between individuals with different resistance alleles.

Dana Nelson, USDA-Forest Service, Southern Institute of Forest Genetics

Personnel working on this project include:

- Southern Institute of Forest Genetics (USDA–Southern Research Station)
  - College Station, TX– Nurul Faridi, Molecular Cytogenetic Lab
  - Saucier, MS– Chuck Burdine, DNA/SSR Lab

- Lexington, KY— Forest Health Research and Education Center
    - Tyler Dreaden-- Plant Pathologist, USFS-SRS
    - Anna Conrad– chemical/biochemical analyses for understanding resistance mechanisms and early screening
    - Shenghua Fan– genomic mapping and rapid cycle breeding
    - Bert Abbott–in charge of biological research
- Clemson, SC
    - Tatyana Zhebentyayev– Phytophthora resistance mapping; GBS markers
    - Rita Costa– Fulbright Scholar, INIVA Portugal; GBS markers, mapping

They are updating the reference map for Chinese chestnut.

- Two reference mapping populations from Anaganostakis and Hebard ('Mahogany' x 'Nanking' n=179 and 'Vanuxem' x 'Nanking' n=158) genotyped with Infinium platform (~1000 new SNPs polymorphic and segregating)
    - Adding to map published by Kubisiak et al (2013)
        - SSRs and SNPs (Golden gate platform)
        - All SSRs and SNPs designed from EST sequences
            - New SNPs also designed from AC (much higher fail rate)
    - 'Vanuxem' showing translocation compared to Mahogany and Nanking
        - Consistent with Nurul's cytogenetic analysis with 'Vanuxem' F_1s

They are using a new reference map to map blight QTLs in various populations

- 'Clapper' B_2 x one Am (Bu3C1C) with no DNA; 76 progeny
    - Results using map made within cross
        - Relatively low polymorphism rate for Clapper (~400 SNPs)
    - One strong, one medium QTL for leaf emergence; LG_L, LG_D
    - No QTLs for blight, amazingly none of the new markers mapped to LG_B (the LG containing Cbr1—from Mahogany F_2 mapping QTL)
- 'Mahogany' B_1 (R4T31 and R4T52 x 10 Am with no DNA; 50 progeny)
    - Data sets complete (about 1000 SNPs polymorphic for each F1 parent)
    - Analyses underway
- 'Nanking' B_1 (KY110 x two Am-- Musik and Mill Creek H, have DNA and genotypes for all three parents; 78 progeny)
    - Finishing data set

'Mahogany' F_2 blight QTLs

- Working with Laura Georgi at TACF
- Expanded population to 214
    - Phenotyped by Fred Hebard
    - Genotyped with Infinium SNPs
    - Genotyping with SSRs
- Need to apply new genetic reference map to best utilize infinium SNPs
    - SSRs are from the published reference map

'Graves' B_3 families in Pennsylvania

- 11 B_3 families (~750 individuals) and B_2 parents genotyped (infinium SNPs)
- Individuals in families phenotyped in field at Penn State

- Preliminary analyzes, not promising for QTL detection– need to redo with new reference map and closer attention to modeling phenotype (better accounting of non-genetic variation)
- Working with Jared Westbrook to complete the multi-family analysis and model the phenotypic variation

Cytogenetics

- Nurul Faridi and Tatyana Zhebentyayev and others
- Using BAC probes from integrated physical-genetic map to identify LGs to chromosomes
  - All 12 identified
  - LG_A and LG_B extensively characterized with 14 and 20 probes, respectively
  - Gene space evenly distributed across the length of the chromosome, unlike cereals
- Major 18-28S RNA locus is on LG_H
  - ~30% of the physical length without genetic markers
- Testing translocations hypotheses using 'Vanuxem' F_1
  - LG_B and LG_E appear negative for translocations

'Mahogany' QTLs in 'Graves' and B_3F_2 population from Meadowview

- Cbr1– LG_B
  - Graves positive for Mahogany allele
  - B_3F_2 sample: 59 -/-; 24 +/-; 1 +/+
- Cbr2– LG_F
  - Graves positive for Mahogany allele
  - B_3F_2 sample: 80 -/-; 1 +/-; 0 +/+
- Cbr3– LG_G
  - Graves positive for Mahogany allele
  - B_3F_2 sample: 41 -/-; 42 +/-; 4 +/+

Ongoing work includes:

- Complete QTL mapping
  - Reference map and QTL maps with Shenghua Fan
  - 'Mahogany' F_2 QTL with Laura Georgi, Fred Hebard, Jared Westbrook
  - PA B_3 families with Jared Westbrook, Sara Fitzsimmons
- Establish SSR screen for 'Mahogany' and other Chinese alleles (haplotypes) in Cbr1, Cbr2, Cbr3
- Complete FISH based karyotype; define translocations
- New collaborations through Forest Health Research and Education Center at the University of Kentucky

John Carlson and Nathaniel Cannon Schatz Center for Tree Molecular Genetics, Penn State University (submitted report)

The Chestnut Genome Sequencing Project. The Chinese chestnut genome sequencing project was initiated with support from The Forest Health Initiative (http://foresthealthinitiative.org/), with subsequent improvement projects supported by The American Chestnut Foundation (TACF) and the Schatz Center at Penn State. Participants in the

project include John Carlson (PI), Charles Addo-Quaye, Nathaniel Cannon, Lynn Tomsho, Daniela Drautz, Lindsay Kasson, Tyler Wagner, Nicole Zembower, Abdelali Barakat, Richard Burhans, Webb Miller, and Stephan Schuster at Penn State University, with Steven Ficklin, Chis Saski, and Bert Abbott at Clemson University, Margaret Staton at the University of Tennessee, Dana Nelson of the USDA Forest Service, and Fred Hebard and Laura Georgi of TACF.

Over the past year, the assembly of the Chinese chestnut cultivar 'Vanuxem' genome has been significantly improved. The current status of the genome sequence is a set of 14,358 scaffolds representing 784Mb of genome sequence, or app. 98% of the estimated genome size (improved from 724.4 Mp in 41,270 scaffolds in our last report). The size of the largest individual scaffold is now 3.17Mb, with average scaffold size of app. 55Kb (improved from N50 of 39.6Kb and largest scaffold of 429 Kb in 2014). Another major step forward has been the anchoring of almost 90% of the genome scaffolds to the genetic map, using the chestnut integrated genetic-and-physical map and the BAC-end sequences covering the physical map of cv. Vanuxem. This allowed us to assemble the scaffolds into a set of 12 pseudo-chromosome sequences, covering the majority of positions along the 12 linkage groups. The draft pseudo-chromosomes allow the physical position of all genetically mapped sequences and the genetic map position of sequences on the pseudo-chromosomes to now be estimated.

Margaret Staton's group in Knoxville upgraded the website for the genome and QTL assemblies to JBrowse format. (http://www.hardwoodgenomics.org/content/tools). The scaffolds, gene models, predicted transcripts and predicted proteomes are all still available to the community to download at the hardwood genomics website. The genome is also now present in the NCBI database. In addition, Meg's group over the past year also conducted an intensive, manual annotation of genes in the QTL assemblies, which are also represented in the browser.

The Carlson group obtained 'resequencing' depth (app. 10X) data for the following set of species and genotypes from CAES and TACF orchards: 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 BC_3 *C. dentata* x *C. mollissima* parental genotypes - B3119 and B3176. We have aligned these sequences to the reference Vanuxem genome to assess within and between species sequence variation in chestnut (refer to Cannon et al poster).

The Staton group conducted detailed variant calling against the 'Vanuxem' reference genome using the above resequencing data from Penn State for the Chinese genotype 'Nanking', the American genotype 'Alex R', the American genotype 'Ellis 1' from SUNY, and the American genotype 'GMBig'. A set of 11,194,851 SNPs was obtained along with a smaller subset of potentially diagnostic 714,039 SNPs supported by sequencing from all three American genotypes. These can be obtained from Dr. Staton as Excel spreadsheets. The SNPs will also be made available online as part of the new chestnut genome browser v1.1.

Work in the coming year will focus on:

- Validate and improve the Chinese chestnut pseudochromosome sequences using very long genome sequences produced from the PacBio single molecule sequencing technology (proposal submitted by Hebard *et al*. to the USDA AFRI program).
- Prepare draft genome assemblies for American chestnut genotype GMBig, Chinese chestnut genotypes 'Nanking' and 'Mahogany', and backcross parent genotypes 'Clapper' and 'Graves' (proposals submitted by Carlson and Westbrook)
- Obtain deep RNA sequence data from several tissues of Chinese chestnut cv. 'Vanuxem' to refine the identification and annotation of genes in the reference genome.
- Submit refereed journal article on Chinese chestnut reference genome (in preparation.
- Host the 2015 annual meeting of The American Chestnut Foundation on October 23 and 24, 2015, to update the TACF membership on the status of and discoveries from chestnut genomics. The meeting will include a workshop that brings together chestnut genomics researchers and TACF members to begin the process of integrating tools from genomics into the breeding and reforestation efforts.

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, New Mexico State University

Controlled induction of hypovirus. There are a number of changes that occur after a hypovirus begins to replicate in a virulent isolate of *C. parasitica*. There is altered colony morphology, reduced radial growth rate, reduced pigmentation and reduced sporulation. Dawe focused on a paper by a former student, Karyn Willyerd, an undergraduate student at the time; she identified a promotor that was controllable by the presence or absence of copper in the medium. She published a paper, "Controlled Gene Expression in the Plant Pathogen *Cryphonectria parasitica* by Use of a Copper-Responsive Element". Her steps were:

- Create a suitable vector that will allow control of initiation expression.
- Clone entire viral genome from existing plasmid pLDST.
- Transform into fungal cells under repressing conditions.

Using copper and a copper transporter, by continually transferring the fungus, it becomes whiter in phenotype. Expression of the virus can be turned on or off, albeit with a limited change in phenotype. The promoter that is used comes from a copper transporter, but the promoter addition is initially not very white. If they are continually subcultured, the phenotype becomes whiter.

Dawe asked the question, why is phenotype feeble? There are a few possibilities:

- The viral genome is not accumulating to sufficient levels for full phenotype.
    - Viral genome is only partially expressed.
    - Virus is fully expressed but not able to initiate replication.
- Tests conducted
    - Perform in the presence of expressed viral protein p48
    - Presence of accumulating viral dsRNA

- Transmission of phenotype via anastomosis
- Reversion of phenotype to wild type when moved from inducing to repressing conditions
- Phenotype without RNAi
- Presence of viral transcript
- Presence of viral proteins

Dawe introduced viral protein p48 via anastomosis to determine if the hypovirus is struggling to initiate replication. While slightly better, it didn't seem that p48 was the answer. The hypovirus genome was not detectable in pRP1-713 transformants. Copper of BCS in medium does not affect anastomosis or transfer of hypovirus between Ep 713 and Ep 155. BCS-grown transformant reverts on copper medium and does not transfer hypovirus. All this indicates that viral replication is not occurring.

Another test used strains from Don Nuss' lab generated by Gert Segers, in an attempt to understand RNAi. Strains were deficient for the ability of fungal defense against viral replication. When the fungal defense system is removed, the result is an extremely debilitated phenotype. Dawe's construct was put into those strains with no positive results of dsRNA accumulation. Detected transcript in the transformant appears to be at a much lower level than in Ep 713.

To recap the tests:

- Perform in the presence of expressed viral protein p48 (modest improvement in phenotype)
- Presence of accumulating viral dsRNA (No)
- Transmission of phenotype via anastomosis (No)
- Reversion of phenotype to wild type when moved from inducing to repressing conditions (Yes)
- Phenotype without RNAi (Unaffected)
- Presence of viral transcript (5' to 3' ends confirmed)
- Presence of viral proteins (in progress)

Evidence points to inducible hypovirus transcript, which results in hypoviral protein production leading to phenotype, but not hypovirus genome replication. Why is there no genome replication? It is likely due to extra 30 nt 3' to the UTR or an artifact from cloning.

Donald Nuss, University of Maryland, Institute for Bioscience and Biotechnology Research, Shady Grove Campus

Super hypovirus donor strains of *C. parasitica* (with Dong-Xiu Zhang and Diane Shi*)*. This task has now been accomplished. Viruses that reduce virulence of *C. parasitica* on chestnut have an exclusive intracellular replication cycle; the way they get around is via anastotmosis and transmission of virus particles. Viruses can be transmitted for biocontrol but the caveat is that the two strains have to be compatible. If they are not compatible, the virus is not transmitted. The system that controls fusion is the vegetative incompatibility system, found in most filamentous fungi. It is controlled in *C. parasitica* by genes at six loci. The *vic* locus is the position of vic genes on the *C. parasitica* chromosomal DNA. There are two alleles

at each *vic* locus—two forms, either allele 1 or allele 2. For the standard reference (virulent) strain, Ep155, the *vic* loci are designated as follows:

vic loci	1234-67
Ep 155	2211-22

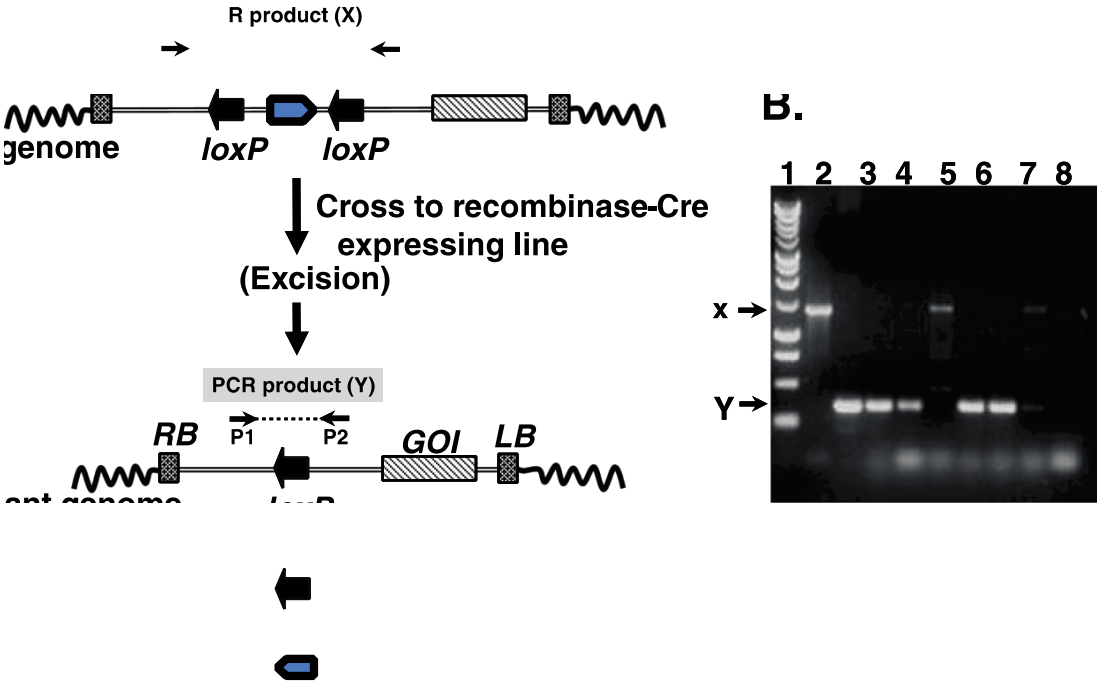
With six genes, $2^6 = 64$ different *vic* genotypes are possible. (Potentially, there are additional *vic* loci that have not been identified genetically or molecularly yet). Strains that have the same alleles at all six *vic* loci are compatible. Strains that have different alleles at any of the *vic* loci undergo an incompatible reaction that restricts virus transmission. Difference at vic-4 does not restrict virus transmission, probably because the programmed cell death occurs more slowly allowing adequate time for cytoplasmic exchange. Milgroom and Cortesi showed that the reactions are asymmetric. An example is vic1. If allele 1 is the recipient, there is good transmission. If allele 2 is the recipient, virus transmission is very low, probably related to the speed at which cell death occurs. Over the last few years, the six *vic* loci that were detected genetically have now been identified molecularly.

A number of genes at the *vic* loci have been disrupted. Most *vic* genes appear to be dedicated to allorecognition with protein features such as HET-domain, sec9-like features. If the genes were disrupted independently, the affects were asymmetric. This indicates that the genes, from the beginning, were recruited to this self-recognition system and they don't seem to have any other functions. The possibility exists for the development of a super hypovirus donor strain that is created by disrupting appropriate genes at the five *vic* loci that restrict hypovirus transmission.

There are complications for making a super donor strain:

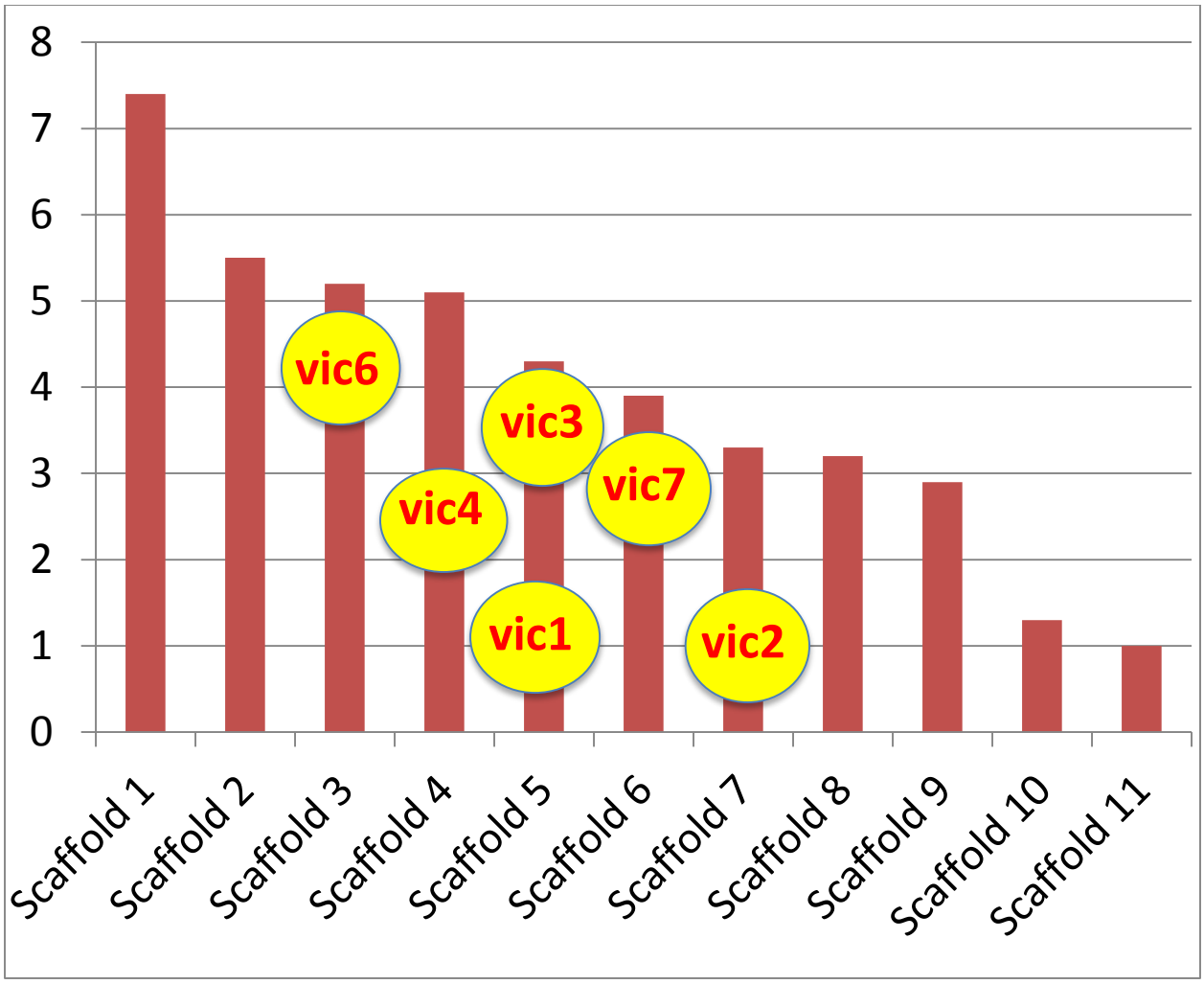
- Need to disrupt genes at five loci and there are only two really good selectable marker genes available—hygromycin resistance and neomycin resistance.
- Use of antibiotic resistance selectable marker genes would likely complicate obtaining permission from EPA and USDA for general use of the super donor strain.

The solution was to adapt the Cre-LoxP recombination system (from a bacteriophage) to delete the appropriate genes at each of the identified *vic* loci and recycle selectable markers to produce a strain that can donate hypovirus to any *vic* genotype and that will be free of any selectable markers that might complicate release of the strain (Zhang et al., *Fungal Genetics and Biology*, 61, 1-8, 2013). In the cartoon below, a selectable marker gene (SMG—ie neomycin), flanked by 27-loxP nucleotides, is excised when a cre-recombinase is added. The SMG is then recycled out, leaving the 27-nucleotide LoxP site.



Anastomosis was used to donate the cre-recombinase transiently. During anastomosis, mitochondria and enzymes are transmitted during cytoplasmic exchange and a small area of hyphae from the recipient strain can be excised and cultured. Resulting conidia are single spored and plated onto media containing particular antibiotics to select isolates that have lost the selectable antibiotic marker. That marker can then be used to knock out the next gene of interest.

Construction of super hypovirus donor. The *vic* genes are located on the scaffolds as show in the following histogram.



The *vic* loci are located on different scaffolds, some are chromosome-size (Mb on above graph). The location of the *vic* genes are on known scaffolds. The genes are unlinked and on different chromosomes. To start the process, Ep 155 was chosen with its known genotype and genes were chosen that are good donors (as opposed to recipient).

Ep 155 (2211-22)
vic1-2, vic2-2, vic3b-1, vic4-1, pix6-2, vic7a-2, ku80

↓ $\Delta ku80^{ben}$

DK80
vic1-2, vic2-2, vic3b-1, vic4-1, pix6-2, vic7a-2, $\Delta ku80^{ben}$

The ku80 strain (ku80 gene was knocked out with a benomyl marker) is important in non-homologous recombination, allowing ku80 strain to undergo non-homologous recombination to knock out specific genes. The *vic1a* gene was knocked out (with neomycin flanked by the LoxP sequences. After adding cre, the first gene was successfully knocked out with the

neomycin marker removed. vic3b, a good donor, was also disrupted as was *pix*6-2. *vic*7a was disrupted leaving all genes disrupted, leaving a quadruple mutant (with all markers removed).

*vic*2 is a strong *vic* locus. It has a fast programmed cell death profile. It has a patatin-like gene (that can be knocked out, making it a good recipient but not a good donor). Nuss tried very hard to knock out a second polymorphic gene (secretory protein) but he was unsuccessful. This suggests that *vic*2 is the only *vic*-related gene that seems to have a central function—it is an essential gene that cannot be disrupted. Thus, they were unable to make a *vic*2 mutant that was a good donor. Nuss resorted to the use of classical mating and segregation to move the *vic* mutations into *vic*2-1 genetic background and restore the wild-type ku80 gene. The quad mutant (2211-22) (MAT-2) was crossed with Ep 146 (2112-11), a brown pigmented isolate (MAT-1). Ascospore progeny were screened (n=840) first by virus transmission from EU55 (1221-22)/pLDST and then by PCR resulting in 5 super donor candidate strains, 4 brown and 1 orange and 4 MAT-1 and 1 MAT-2. Three were vic2-2 and two were vic2-1, as shown below:

#82: Δvic1-1, vic2-1, Δvic3b-1, vic4-1, Δpix6-2, vic7a-2, ku80, Brown, MAT-2
#328: Δvic1-1, vic2-2, Δvic3b-1, vic4-1, Δpix6-2, vic7a-2, ku80, Brown, MAT-1
#684: Δvic1-1, vic2-2, Δvic3b-1, vic4-1, Δpix6-2, vic7a-2, ku80, Brown, MAT-1
#752: Δvic1-1, vic2-2, Δvic3b-1, vic4-1, Δpix6-2, vic7a-2, ku80, Brown, MAT-1
#782: Δvic1-1, vic2-1, Δvic3b-1, vic4-1, Δpix6-2, vic7a-2, ku80, Orange, MAT-1

Super donor candidate strains exhibited normal growth, colony morphology and virulence on dormant chestnut stems. Sporulation and mating competence tests are being completed; however, all preliminary results indicate normal reproduction capabilities. Virus transmission is highly superior over parental strains. Three strains (82, 328 and 782) were chosen for further characterization.

Next steps:

- Publication
- Permission from EPA for field trial to test virus transmission in the field
- EPA registration as a biopesticide
- Potential applications:
    - Maintain surviving American chestnut tree genotypes adapted to local ecosystems for use in the backcross breeding efforts—mother trees.
    - Treat blight in orchards containing blight susceptible cultivars.
    - More effective integration of hypovirulence and host resistance for chestnut reforestation efforts.

William MacDonald, 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 3,467 cankers have been identified in the 12 plots. Two hundred, eighty-one cankers on living trees were sampled in July 2015; 78 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 2014, 35% of the original stems in the Disease Center plots remained alive compared to 18% and 22% 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.
- Chestnut sprout populations have increased significantly as the mortality of the original stems has resulted in additional light reaching the understory.
- Vegetative compatibility type WS-1 continues to be the dominant vc type in the stand although its frequency has decreased from 100% in 1995 to 74% in 2014. WS-2 and WS-3 were found at rates of 4% and 7%, respectively.

Tomah, WI Chestnut Stand. An 80-acre woodlot in Tomah, WI (approximately 30 miles east of West Salem) contains a number of large American chestnut trees and many naturally-occurring seedlings. The stand was blight free until 2011. Some mature trees that died from chestnut blight have been harvested by the landowner. Nine random cankers were sampled in July 2015; all *C. parasitica* isolates had a virulent morphology. An isolate from each canker was paired with the three main West Salem isolates (WS-1, WS-2 and WS-3). Tomah 3 was compatible with WS-1; all others were incompatible. Isolates were genotyped: Tomah 1, 2, 4, 5, 6, 7, 9 were EU 17. Tomah 3 was EU 52 and Tomah 8 was comprised of two genotypes, EU 52 and EU-28. WS-1 (Euro) was paired with all Tomah isolates and none were converted.

B_3F_3 Planting at the University Forest, Morgantown, WV. One hundred advanced backcross seedling were planted in April 2015 at the University Forest near Coopers Rock in Preston County. WVU forestry students, members of the Urban Forestry Club, helped with the planting.

Multilocus PCR Assays Elucidate Vegetative Incompatibility Gene Profiles of *Cryphonectria parasitica* in the United States (in conjunction with Matt Kasson and Dylan Short). In order to effectively determine the vegetative incompatibility genetic structure of *C. parasitica* in field populations, PCR primer sets were designed that selectively amplify and distinguish alleles for each of the six known di-allelic *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 U.S. revealed 39 unique *vic* genotypes and generally good agreement between PCR and tester strain co-culturing assays in terms of *vic* diversity and genotyping. However, incongruences were observed for isolates from multiple locations and suggested that the co-culturing assay can overestimate diversity at

the six known *vic* loci. 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.

Cameron Stauder, West Virginia University

Comparisons of host susceptibility to isogenic virulent and hypovirulent strains of *Cryphonectria parasitica* among *Castanea* hosts and plant tissue types. This study was designed to: 1) compare the level of host susceptibility to *Cryphonectria parasitica* among American, Chinese, European, and three generations of American x Chinese hybrids (B_2F_2, B_2F_3, and B_3F_2) when inoculated with virulent and hypovirulent inoculum sources; 2) to utilize a leaf assay procedure (Newhouse et al., 2014) as means of resistance screening on a larger scale; and 3) compare the effect of hypovirus species (CHV1 and CHV3) on a single, isogenic strain.

For the host susceptibility comparison, 111 trees were inoculated with a virulent strain designated "Weekly", a CHV1 hypovirulent Weekly, and a sterile water agar control. Resultant cankers are to be measured for area and assessed for sporulation at two-month intervals over one-year period. The first two-month measurement revealed few significant differences in host susceptibility based on canker area and sporulation. The additional two objectives for this study have not been completed to date, but efforts will be resumed winter/spring of 2016.

Table 1. Average canker area (cm^2) at two months after inoculation

Species/Hybrid	Inoculations			Stroma/cm^2
	Weekly	WK CHV1	Control	
American	61.13	10.94	4.92	0.36
B_2F_2	52.40	10.87	5.58	0.29
B_2F_3	60.29	13.58	4.40	0.37
B_3F_2	60.00	10.91	6.43	0.46
Chinese	40.37	10.16	4.64	0.27
European	50.08	8.21	4.19	0.38

Leaf assay source: Newhouse, A. E., J. E. Spitzer, C. A. Maynard, and W. A. Powell. 2014. Chestnut leaf inoculation assay as a rapid predictor of blight susceptibility. Plant Disease. 98(1):4-9.

Matthew Kolp, Michigan State University

Intra-canker variability for secondary fungi. (In cooperation with Bill MacDonald and Mark Double, WVU; Eric Eager and Anita Davelos Baines, University of Wisconsin, La Crosse). Investigations into microorganisms inhabiting chestnut blight cankers continues at five American chestnut populations in the northern Lower Peninsula of Michigan and at a stand of chestnut in West Salem, WI. At four of the five Michigan chestnut populations sampled in 2014, cankers rated 'girdling' contained more virulent strains of *Cryphonectria parasitica* than those rated 'non-girdling'. At West Salem, WI, girdling cankers contain more hypovirulent *C. parasitica* than non-girdling cankers, but this may be due to an ongoing treatment program involving hypovirus application made directly to girdling cankers and not to non-girdling cankers. Further, non-girdling cankers contained more secondary, non-pathogenic fungi (Non-CP) than girdling cankers except at one site in Michigan (County Line), while the frequency of

hypovirus between the two canker types was variable. Species-specific effects of certain Non-CP associated with girdling and non-girdling cankers are still being analyzed. It was found that the communities of Non-CP within cankers were different among the six chestnut populations. Ultimately, the goal is to determine how certain Non-CP species that act as antagonists towards *C. parasitica* can be used in concert with hypoviruses to best slow canker expansion and prevent girdling of chestnut trees.

Josh Springer and Dennis Fulbright, Michigan State University

Hypovirus Inoculations. In May 2015, a plot with the European X Japanese hybrid cultivar 'Colossal' was established to determine if a mixture of five vc types infected with the hypovirus CHV3-GH2 could manage chestnut blight cankers initiated by a single vc type on mature branches. Virulent cankers were initiated with MSU orchard vc type 1, and 8 weeks later, the small, but expanding cankers were treated with a single strain of vc type1 infected with CHV3-GH2 or a mixture of 5 vc types including vc type 1. Obviously, the hypovirulent treatment mixture cut the amount of the matching strain by 80 percent when compared to the single strain treatment. In addition, the treatments were made with full strength and at 50 percent strength. The cankers were treated by scratching the cankers with a device where wood screws were imbedded in a wooden handle. The data were recorded 8 weeks after treatment. All non-treated cankers were given the maximum rating of a 4 indicating little or no callus, and all treated cankers were rated a 1 or 2 indicating the treated cankers had wound tissue and callus present.

In orchards, our goal is to not allow the cankers to become large. Therefore, a major educational program is now in place where growers are being asked to survey their orchards for chestnut blight. Blight is found mostly on the European X Japanese hybrid cv. 'Colossal', the most common blight-susceptible tree planted in Michigan. Some interesting general information was list listed below in 2014 and new information has been added:

1. A mitochondrial hypovirulent strain was isolated in one orchard on a surviving 'Colossal' tree (80 percent girdled). The hv strain was found in the non-girdled tissue. The tree was treated with a hypovirus-containing strain. This tree is still alive, has production, and has produced more callus tissue.
2. Orchards established near sources of blighted trees have more blight than orchards not planted near blighted trees. However, these non-orchard infected trees near orchards have not been shown to be the main source of C.p. strains found in the orchards. Based on vc types in the orchard and in the nearby stand of trees, there are few matches.
3. Chain saw wounds have been successful in treating trees with hypovirulent strains.

Registration of GH2 hypovirus as a biological control. With the expanding chestnut industry in MI, the importance of treating cankers is important to keep an orchard productive. The importance of treating orchards is seen in the following table.

Treatment	Avg. increase in trunk size 2011-2012	Avg. increase in trunk size 2012-2013
Disease-free	1.21 cm	1.76 cm
Hypovirus inoculated	0.93 cm	1.40 cm
Blight infected	0.72 cm	1.24 cm (2 trees died)

There is an effort to get hypovirus GH2 registered as a biopesticide through IR-4, a USDA, EPA program used to collect and evaluate data on residue and efficacy of minor-use pesticides. Will the new pesticide or biopesticide harm the environment or those that consume a product form a vegetable, fruit or nut, etc? The registration of GH2 as a biological control is necessary because:

- Cannot use, as food, nuts from trees in the year of hypovirulence application (all work done in orchards is research and not marketable technology until registration)
- Growers can lose significant revenue ('Colossal' *e.g.*).
- If a tree is treated in 2015 and produces about 80 lbs, then the grower loses ~$200 (70% of a healthy tree productivity but if no hypovirulence is used, the yield is zero).
- If left untreated, a tree can quickly succumb to chestnut blight and lose all revenue for many years, especially if the tree is productive and >15 years old.

Their goals are:

- Show, yet again, that hypovirulence works, but more evidence is needed in orchard situations in a better designed study.
- Understand if cankers should be treated with 1 GH2 infected vegetative compatibility (VC) type OR a mixture of common GH2 infected VC types (again, unpublished work).

This could be important for other chestnut 'combinations' that are released into the field.
Study plan:

- Virulent and hypovirulent controls
- Full concentration of common VC type infected with GH2 (40g dry weight/200mL PDA)
- ½ concentration of common VC type infected with GH2 (20g dry weight/ 200mL PDA)
- Full concentration of 5 most common VC types in MI orchards infected with GH2
- ½ concentration of 5 most common VC type infected with GH2
- Why a mix of VC types infected with GH2?
    - Don't need to match VC type

To conduct the experiment, 40 individual branches on JXE hybrids, 'Colossal' were inoculated with: 20 branches of V, ½1, full1, HV
20 branches of V, ½5, full 5, HV
All wounds were created with a 10 mm cork borer. All cankers were allowed to develop for 5 wk before further treatment. Cankers were scratched to expose living tissue. Inoculum was applied in a syringe (5 cc) of either 1 or 5 vc groups infected with GH2. After scratching and application of HV material, cankers were wrapped with laboratory absorbent pads.

Canker evaluation 5 wk after treatment were rated on a 1-4 scale:

1. Wound swollen with very few (1-5) stroma
2. Wound with moderate swelling with some stroma (6-15)
3. Wound with no or little swelling and many stroma (>16)
4. Wound sunken with many stroma and canker enlargement

Preliminary results:

	Conc.	*Ratings*			
		1 Good	**2**	**3**	**4 Bad**
Control					
V		2	1	6	21
HV		10	25	2	2
Treated					
1 VC	**Full**	5	14	1	0
1 VC	**Half**	5	13	2	0
5 VC	**Full**	9	11	0	0
5 VC	**Half**	10	10	0	0

<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

Fred Hebard, The American Chestnut Foundation, Meadowview

Evidence that a single gene controls Phytophthora root rot (PRR) resistance in 'Graves' backcross. This work was started by Joe James and Steve Jeffers in 2005; they rated trees that had died of PRR on James' farm. Jeffers designed all the protocols and James implemented them. Paul Sisco supplied genotypes early on in the experiment, and he recruited Hebard to supply material from the TACF network. The PRR picture began to be clarified around 2010-2011 following testing of B_2s and B_3s. The testing is done in large tubs that hold 500 liters of soil mix. The seedlings are inoculated and the tubs are flooded for two days to aid in *Phytophthora* establishment.

Typical severity of Phytophthora root rot (PRR) in susceptible, resistant and intermediate resistant chestnut.

Resistance	Cross Type	Phytophthora Root Rot Severity			
		None	Slight	Moderate	Dead
Susceptible	American	0	0	2	81
Intermediate	B_1 HB1	0	2	23	40
Intermediate	B_1 HB2	1	6	42	30
Resistant	Chinese	66	16	2	1

Hebard concluded that resistance is incompletely dominant and the best partition of classes is between live and dead trees. With more accurate classification of segregants, co-dominant classification might be useful for F_2s.

In a test comparing twelve B_2 parents with 'Clapper' as the source of resistance, eleven of twelve were susceptible. In a similar test comparing 'Graves' and 'Mahogany', only eight were susceptible. In a test of B_3 parents with 'Graves' as the source of blight resistance, ten of seventeen were susceptible. Hebard's inferences were that 1:1 segregation of resistance in

backcross suggests it is controlled by a single factor. 'Clapper' progeny do not have PRR resistance, unlike 'Graves' progeny. More F_2 populations are needed from controlled crosses.

From a study that examined the severity of PRR in a 'Mahogany' B1 mapping cross, HB2 in two years of testing (compared to HB1) indicated that the American parent had an effect on PRR severity, at a low level of significance. The HB2 cross, a B_1, showed more resistance than most 'Graves' and 'Mahognay" B_3s and B_4s as indicated by progeny test. There were more trees in the moderate class and fewer in the dead class in 2012 compared to 2011.

Hebard concluded that PRR resistance is prevalent in 'Graves' B_3F_3s. The resistance in some families appeared to be better than the intermediate levels found in trees heterozygous for resistance, suggesting some of those trees were homozygous for resistance. The straight B_3 grandmothers of the B_3F_3s did not have PRR resistance, so they must have inherited it from their parents and grandfathers.

- Resistance to Phytophthora root rot incited by *P. cinnamomi* is controlled by a single, incompletely dominant gene in the 'Graves' first backcross from Chinese to American chestnut.
- For 'Graves', we expect the allele for PRR resistance to be occurring at a frequencies of 1 in 4 at straight B_2 and 1 in 8 at straight B_3, with no selection. Observations fit those expectations.
- If so, in subsequent filial generations (B_3-F_2, etc), trees homozygous for PRR resistance would occur at frequencies of $(1/8)^2 = 1/64^{th}$, and heterozygotes at frequencies of $2x(1/8)x(7/8) = 14/64^{th}$, by the Hardy-Weinberg principle. The data are compatible with this hypothesis.
- Breeding orchards composed of trees homozygous for PRR resistance, and reasonably homozygous for blight resistance, could be obtained at the B_3-F_4 generation, in manageable populations.

It is thought that this plan could be implemented in 10 years or less. Currently, screening for PRR resistance in B_3-F_3 seedlings is being optimized.

Steve Jeffers, Clemson University

Update on research on ink disease of American chestnut. Jeffers is continuing to screen hybrid American chestnut seedlings for resistance to Phytophthora root rot, PRR (=ink disease) Jeffers and MS graduate student, Suzette Sharpe, are investigating the diversity of species of *Phytophthora* associated with American chestnut—this is in conjunction with TACF and the USFS with Stacy Clark. They also are looking at the pathogenicity of *Phytophthora* spp. to American and Chinese chestnut along with the potential variation in virulence of *P. cinnamomi* and *P. cambivora* on American chestnut. Finally, he is working on molecular genetics of resistance in cooperation with Tatyana Zhebentyayeva.

Ink disease was first described in Europe on *C. sativa*. It initially was caused by *P. cambivora*. Later, *P. cinnamomi* also was shown to be pathogenic but more aggressive than *P. cambivora*. Between 2001-2005, two additional species were implicated, *P. cactorum* and *P. citricola,* but they were not as virulent as *P. cambivora* or *P. cinnamomi*. In the US, the only

species found for more than 80 years is *P. cinnamomi*. In 1932, the pathogen initially was thought to be *P. cambivora* but it was identified in 1945 as *P. cinnamomi*. It is speculated that the pathogen was introduced into the US in late 1700s or early 1800s through one of the ports in the southeastern US. Trees in the southern range started dying in the 1800s and ink disease became widespread at lower elevations in southeastern states. The pathogen is not a true fungus but an Oomycete. The vegetative cells have a 2n nuclear state and the cell walls are composed of cellulose. The populations in the US is thought to be clonal with the A2 mating type predominating.

This disease on *Castanea* spp. is caused by several species of *Phytophthora* worldwide. Currently, Jeffers is focused on resistance in hybrid American chestnuts seedlings caused *by P. cinnamomi*. Isolates of *P. cinnamomi* are known to vary in virulence on other host plants. Resistance to one species may or may not provide resistance to other species. Eventually, the goal is to screen for resistance to all species of *Phytophthora*, using the most virulent isolates.

James and Jeffers screen seedlings of hybrid American chestnuts in Seneca, SC in 150 gallon watering tubs as shown below.

Chestnut seeds are geminated over the winter and planted in rows 1-1.5" apart. They use five seeds/family/replicate; one tub = one replicate. Both American and Chinese chestnuts are used as positive and negative controls, respectively. They use vermiculite moistened with V8 juice broth that has been colonized by *P. cinnamomi* as inoculum. Tubs are flooded with water for 6-8 hours once or twice during the growing season to promote infection and disease development. Symptoms are generally seen within two weeks. Seedlings are rated in late December for symptom severity using a 0-3 scale: 0=healthy plant; 1=lesions on feeder roots; 2=lesions on tap root; 3=dead plant.

Diversity of species *Phytophthora* associated with American chestnut. This work is conducted in collaboration with Tom Saielli (TACF) and Stacy Clark (USFS). The objective is to determine if species of *Phytophthora* other than *P. cinnamomi* are associated with American chestnut. The approach is as follows:

- Isolate species of *Phytophthora* from plant and soil samples collected from forest sites and potential chestnut-growing sites in eastern US
- Isolation from diseased roots on selective media
- Bait bioassays for soil samples
- Results:
    - Research is being conducted by a MS student—focusing on samples from Stacy Clark
    - 300-400 plant and soil samples have been received and processed

- *P. cinnamomi* has been isolated most frequently with variation in colony morphology recognized.
- *P. cambivora* or a genetic variant was isolated from samples collected in several southern states.
- *P. heveae* was isolated from roots and soil samples from several sites in NC.
- *P. drechsleri* was isolated from plants at one site in Seneca, SC.
- Several unidentified isolates also have been recovered.
- Representative isolates from all four species and the unidentified group will be characterized.
  - Morphologically: growth habit, spore types/features
  - Physiologically: mating type, fungicide sensitivity
  - Molecularly: DNA sequence and RFLP fingerprints
- Geographic distribution of each species will be determined but there does not seem to be any correlation between nursery where seedlings were grown and Stacy Clark's forest plantings.

Virulence of *P. cinnamomi* and *P. cambivora*

The objective was to determine if isolates from different geographic sources vary in virulence on American chestnut. The approach was:

- Experiment not started yet—2 trials are planned
- Inoculum is growing in the incubator—27 isolates
- *P. cinnamomi*: 5 sources with 3 isolates/source
- *P. cambivora*: 4 sources with 3 isolates/source
- Procedure is similar to that used for pathogenicity

Paul Sisco, The American Chestnut Foundation, Asheville

Sisco presented five goals of the Joe James/Steve Jeffers Tub Tests for PRR Resistance 2004-2015:

- Goal 1: Identify PRR resistant BCF_2 trees for a seed orchard at the James Farm in Seneca, SC
- Goal 2: Identify advanced BC trees with PRR resistance for chapter breeding programs
- Goal 3: Identify BCF_2 trees with PRR resistance for TACF-Meadowview
- Goal 4: Screen GMO trees for PRR resistance
- Foal 5: Screen controlled early-generation progenies to identify loci associated with PRR resistance

Sisco indicated that he wanted his comments to be brief to allow more time for Tatyana Zhebentyayeva and Rita Costa from Portugal, who are working together to garner hard molecular data to elucidate the genetics of *P. cinnamomi*. Sisco commented on the James/Jeffers tub study indicating that the PRR work is a team effort.

For genetic work, all seedlings are sampled by removing leaves for DNA extraction prior to inoculation. Sisco began working on PRR in 2005. As the former southern regional science coordinator for TACF, he started getting backcross seeds from the south where PRR was a big problem. Initially, they tested a lot of 'Clapper' trees, because Tennessee and North Carolina chapters were using 'Clapper' backcrosses then. They were testing a lot of 'Clapper'-derived

trees but not getting much PRR resistance. They had tested fewer 'Graves'-derived trees, but Hebard suggested in 2009 that there was a single factor for PRR resistance segregating in these 'Graves'-derived BC trees. They spent an additional three years to test 'Clapper'-derived trees to verify that they did not harbor PRR resistance.

Goal #1: The first goal was for Joe James to have a seed orchard with trees that have resistance to both blight and PRR. A decade ago, James felt that TACF wasn't doing enough relative to PRR resistance. That was the initial goal—to provide seedlings that had resistance to both diseases. James has planted a lot of chestnuts on his 200 acre farm in Seneca, SC and Sisco is worried that many of the trees have only moderate resistance to PRR and chestnut blight. They survive but they are not as good as Chinese.

Goal #2: Once Hebard had reported that 'Graves'-derived trees had some PRR resistance, Sisco began to chart out BC_2 and BC_3 trees that were 'Graves' derivatives that had been selected for blight resistance. Some of those trees also seemed to have PRR resistance on the basis of progeny tests, and the southern chapters began to concentrate their breeding on those trees. Tom Saielli is still using some of these trees in breeding for the southern chapters.

The Tennessee chapter, with the help of Taylor Perkins and Hill Craddock, has set up its own PRR screening. They have five tubs and Craddock has purchased five more tubs for additional testing.

Goal #3: Goal three is to identify BCF_2 trees with PRR resistance for TACF-Meadowview. The Meadowview staff is producing Restoration 1.0 Chestnuts that are sent to members and cooperators, and TACF would like to know if any of the trees in the Wagner and Duncan seed orchards also have PRR resistance. James and Jeffers are doing the same sort of progeny tests to determine this.

Goal #4: James and Jeffers also are screening GMO trees from Scott Merkle (UGA) and Bill Powell (SUNY-ESF). The GMO trees are coming out of culture and they are not very strong and that is causing problems in getting accurate phenotypic data for PRR resistance. However, the GMO trees being screened in 2015 appear to be stronger.

Goal #5: Goal 5 is to screen early-generation progenies generated by controlled pollination to identify loci associated with PRR resistance in *Castanea mollissima* cvs. 'Mahogany' and 'Nanking' and in several cultivars of *C. crenata*. That is the work being conducted by Zhebentyayeva and Costa.

Tatyana Zhebentyayeva, Clemson University

Mapping of resistance to *Phytophthora cinnamomi* in interspecific American/Chinese and American/Japanese chestnut family hybrids. Before the arrival of *C. parasitica*, the southern portion of the American chestnut range was wiped out because of *P. cinnamomi*. Zhebentyayeva showed the 0-3 rating scale for phenotyping based on severity of root rot symptoms (see Jeffers' report). In the initial mapping efforts of interspecific BC1 cross: AdairKY (American chestnut X GL158), 48 individuals were examined. DNA was collected from healthy plants prior to inoculation. A low density map was composed with 203 SNPs on 12 linkage groups. The above-ground phenotyping segregation ratio was 1:1 (27 alive vs 4 struggling/17 dead).

Based on multiple year data for resistance to Phytophthora root rot (PRR), two BC_1 families were selected: HB2-2014 (237 individuals) and NK4-2014 (318 individuals) for high-

throughput genotyping using a genotyping-by-sequencing (GBS) approach. In 2014, a segregation ratio of 1:1 (alive: dead) for resistance to *Phytopthora cinnamomi* (Pc) was found in both families (x^2= 1.52, $p < 0.05$ and x^2= 0.3, p<0.05, $p < 0.05$ for HB2_2014 and NK4-2014 respectively). Phenotypic data are presented in Table 1.

Table 1. Phenotypic data for PRR resistance for the BC_1 hybrid families selected for genotyping-by-sequencing. 0=no symptoms; 1=symptoms only on feeder roots; 2=symptoms on tap root; 3=dead at the end of one season of exposure to *P. cinnamomi*. The screening technique is described in Jeffers et al. 2009.

Hybrid population code-year	Total plants	Root rot symptoms severity classes				Source of resistance
		0	1	2	3	
HB2-2014	237	0	3	106	128	*C. mollissima* 'Mahogany'
NK4-2014	318	2	17	135	164	*C. mollissima* 'Nanking'

DNA extracted using a CTAB protocol by Kubisiak *et al*. (2013) was quantified using Quant-iT™ PicoGreen® dsDNA assay kit (Life technologies) and Synergy H1 microplate reader (BioTek, VT, USA). DNA concentrations were normalized to 10 ng/µl, double digested with Pst+Msp1enzymes and subsequently used for library preparation. Parental DNA was incorporated into 5 random wells across the GBS plates. Sequencing libraries were prepared according to the GBS protocol as per Elshire *et al*. (2011). Paired-end 2 x 125 bp sequencing of 48-plexed GBS libraries was performed at the Medical University of South Carolina, Charleston, on five lanes of an Illumina HiSeq2500 instrument. The workflow for processing reads using Stacks (Catchen et al. 2011) is presented in Fig.1.

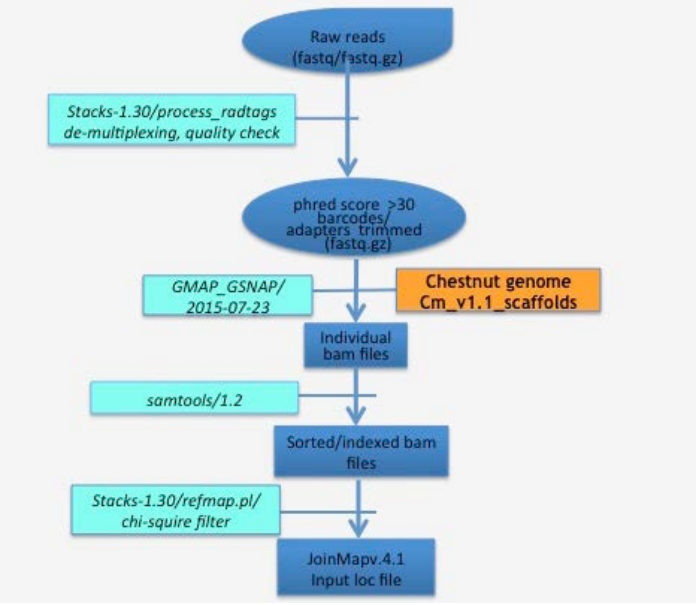


Fig. 1. GBS data processing pipeline.

In total, 1965 million (mln) raw reads were generated for 237 HB2-2014 BC_1 progeny and the female (KY115) and male (AD98) parents. More than 96% of the reads were retained after a check for read quality (QC score >30), intact barcodes and restriction sites. Currently, alignment of individual GBS reads against the reference chestnut genome assembly Cm_v1.1_scaffolds, SNA genotyping using Stacks and linkage genetic mapping are in progress (Table 2).

Table2. GBS data processing statistics for HB2-2014 cross

	HB2-2014 (237 individuals)	Male parent KY115 (P1)	Female parent AD98 (P2)
Total reads (mln)	1880	48.0	37.4
Retained reads (mln)	1815	47.4	36.9
Retained reads (%)	96.5	98.8	98.7
Reads/per individual (mln)	7.5	47.4	36.9
Failed (< 2 mln reads/ind)	13	-	-

Rita Costa, Portugal (Visiting Fulbright Scholar, Clemson University/ Instituto Nacional de Investigação Agrária e Veterinária)

Genetic and genomic approaches for understanding the responses of Castanea spp. to infection by Phytophthora cinnamomi. The Portugal team working on this project includes: Carmen Santos, Helena Machado, Susana Serrazina, Isabel Correia, José Gomes-Laranjo, Filomena Gomes and Rita Costa. Her American colleagues include: Tatyana Zhebentyayeva, Christopher Saski, Chuck Burdine and C. Dana Nelson.

There is genetic diversity of *C. sativa* populations in Europe. According to Mattioni (2008), there are five distinct gene pools of chestnut in Europe. Three are located in Greece, one in the Northwest coast of the Iberian Peninsula (IP) and a large genepool covering the rest of the Mediterranean Basin. Regarding the Iberian Peninsula, the studies performed under the Castanea REG project corroborated the genetic differentiation between Northern and Central Spain, previouly described by Mattionni and revealed for the first time that the most important origins of diversity are located North of IP (Asturias and Galicia) and in Central IP (Trás-os-Montes and Central Spain). TMontes Region, which has been evaluated for the first time, presented the highest number of different genotypes (96 different genotypes in 187 accessions) indicating the importance of this region as a diversity origin. The genetic differentiation between Northern and Central IP can be explained by genetic adaptations to climatic conditions, mainly temperature and precipitation gradient. Southern and Canary islands stands are younger, having origins in both the North and Central regions.

In Portugal, there are about 41,000 ha for chestnut production—about 2 tons/ha. Breeding was initiated in 1948, but the program did not proceed with consistency. Little information was acquired on the genetics of resistance to *P. cinnamomi*. New crosses were started in 2006 supported by molecular tools, mapping and transcriptomic approaches were implemented.

Costa reviewed *P. cinnamomi*. It is an Oomycete, an aggressive primary pathogen with a wide geographical range and nearly 1,000 host species. It has a huge economic impact on

principal food crops like avocados and pineapples. It also attacks *Castanea, Cinnamomum, Coniferales, Ericeae, Eucalyptus, Fagus, Juglans, Quercus* and many ornamental trees and shrubs. American and European chestnut are susceptible while Japanese and Chinese chestnut are resistant to PRR.

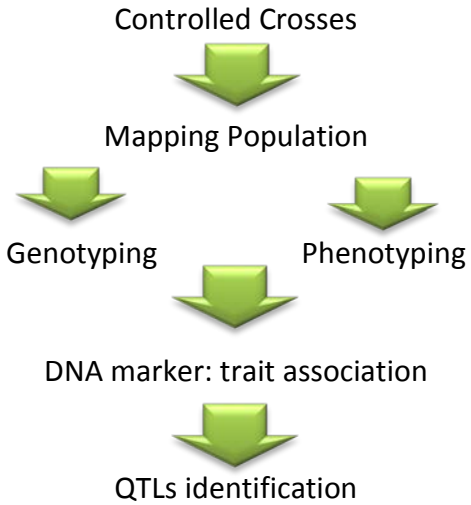
There is molecular evidence for an Asian origin and a unique westward migration of species in the genus *Castanea* via Europe to North America. The phylogeny of *Castanea* was estimated using DNA sequence data from different regions of the chloroplast genome. Sequencing results support the genus *Castanea* as a monophyletic group with *C. crenata* as basal.

Costa started making crosses in 2006 using the model of The American Chestnut Foundation. She used *C. sativa* as a female and *C. mollissima* and *C. crenata* as males. The aim was to develop resources in order to find loci related to resistance to *P. cinnamomi*. She used two approaches: genetic mapping and transcriptomics. The goal was to:

- Develop saturated genetic linkage maps and identify QTLs for resistance;
- Identify candidate genes of resistance with further validation;
- Co-localize the validate resistance genes with QTLs;
- Perform comparative genomics;
- Select markers linked to resistance to perform marker assisted selection;
- Select resistant plant material with commercial potential for rootstocks.

The last goal is very important because, at this time, there is only one commercially available rootstock in Portugal. Looking for additional commercial resistant rootstocks fits with the goal in Portugal with respect to applied research.

Costa's mapping approach is:



There are not many F_1 progenies currently but Costa is repeating crosses with the aim of increasing the number of progeny.

For phenotyping, she screened resistance/susceptibility to *P. cinnamomi* via two tests: root inoculation and excised shoot inoculation. Plants were produced by *in vitro* culture after the root inoculation and shoots from progenies were developed following the excised shoot inoculation. Twenty genotypes were tested and *P. cinnamomi* was evaluated 100 days post inoculation. At the point of death, low-rated plantlets showed typical symptoms of *P.*

cinnamomi: necrotic lesions in roots, which, in some cases, extends to the root collar and shoots, chlorosis and wilting of leaves, die-back of shoots, and plant death. Depending on the genotype, the lesions were either limited and localized at some roots or invasive in the entire roots, causing wilting of the plantlet. All control plantlets survived 100 days after inoculation. No symptoms associated with *P. cinnamomi* were observed in the roots, root collar and shoots of the control plantlets, indicating that cross contamination did not occur. Plantlets with soil infestation started dying in average at 10.25 days after inoculation and continued to die throughout the experiment. The difference of the estimate of days of survival between inoculated and control plantlets was larger for susceptible genotypes and less for the resistant genotypes. Plantlets of the majority of genotypes tested did not survive until the end of experiment (100 days after inoculation). Only 13.14 % of total plantlets with soil infestation survived 100 days after inoculation, corresponding to 7 different genotypes (35%). Each genotype responded differently to inoculation with *P. cinnamomi* and currently six genotypes have been selected for high resistance; those are undergoing micro propagation and acclimatization in order to test their compatibility for fruit production.

In the phenotyping/excise shoot experiment, 63 genotypes were tested during two seasons (spring and fall). Measurements were conducted 5, 7, 9, 12 and 14 days post inoculation with *P. cinnamomi*. Lesion progression rate (cm/day) was calculated. Five days after inoculation, *P. cinnamomi* induced visible necrotic lesions of varying length, limited by a black line developing in the cortical tissues. The lesion length (L) progression rate varied from 0 to 1.48 cm per day in all genotypes and in both seasons.

Also, phenotyping was done for blight resistance to *C. parasitica*. Twelve genotypes (6 resistant and 6 susceptible to ink disease) were tested; 9 replicates of 3 inoculations/3 stems. Lesions were measured at 10, 14, 17 and 21 days post-inoculation and lesion progression (cm/day) was calculated. One genotype (SM08) had small lesions for both *P. cinnamomi* and *C. parasitica.*

For the transcriptomics work, 5-year-old trees were used along with micropropagation plants. Susceptible *C. sativa* (3 clones) and resistant *C. crenata* (six clones) plants were used. Both were inoculated with *P. cinnamomi* and the roots were collected at 48h, 96h and 7 days after inoculation. RNA was isolated to obtain 4 RNA pools. Following 454 sequencing platform, *P. cinnamomi* resistance candidate genes were selected and validated by digital PCR. In summary, 43 new molecular markers were developed in candidate genes.

Genotyping and SSRs and SNPs. From *C. mollissima*, 378 SSRs (from the SIFG group), along with 43 SSRs from *C. sativa* and *C. crenata P. cinnamomi* candidate genes and 2553 SNPs from the *C. mollissima* and *C. dentata* (Fagaceae project) were examined.

For the genetic map and *P. cinnamomi* resistance QTLs:

252 markers mapped/448 marker genotyped
103 Cm RRTs mapped/144 Cm SSRs genotyped
15 New SSRS mapped/21 New SSRs genotyped
134 SNPS mapped/278 genotyped
Total: 74 individuals
16 Linkage groups
2 QTLs
LOD-4

Validation by digital PCR is in progress (assessing plant production by micropropagation) and root inoculation with *P. cinnamomi*. Currently, Costa is working with Tatyana Zhebentyayeva making GBS libraries. She is continuing phenotyping new progenies, designing new markers from *C. sativa* genome, genotyping by sequencing (work being conducted with Tatyana). She hopes to merge and integrate new markers into the linkage map for co-localization of resistance genes with QTL.

Sandra Anagnostakis, The Connecticut Agricultural Experiment Station

Seed orchard: (2007 seed, trees planted in Griswold, CT in 2009)

1. 420 trees that are half-sibs, BC_2 (from Chinese) x BC_3 (from Japanese)
   360 trees that are half-sibs, BC_3 (from Japanese) x BC_3 (from Japanese)
2. Survival in 2015 (7 years old): 176/420 and 83/360
   -Expected survival if there are 3 genes for resistance - 6/420 and 5/360
3. Tests of soil in the orchard revealed very low levels of minerals. Tests also were done on petiole samples of leaves collected from both groups of trees. The leaf results showed extremely low levels of several vital minerals, including boron and manganese which are known to affect disease resistance in plants.

Leaf minerals, ppm:

Element	Normal	Griswold
Aluminum	461.5	316.95
Calcium	7762.75	7950
Magnesium	2306.25	1950
Nitrogen	22646.5	
Phosphorous	2582.25	1650
Potassium	8356.5	7100
Sulfur	1652	
Boron	49.25	17.35
Copper	6	13.7
Iron	57.5	37.5
Manganese	461.75	106.35
Zinc	38	38.2

Half of the trees received soil-applied supplements in Sept. 2015; they were retested after two weeks. Additional supplements and testing will follow.

Hybrids planted and monitored recently

1. Fairfield, CT, open spaces (Fairfield Garden Club), 2010 seed
   - 100 back-crossed, timber-type chestnut trees planted 2012, distributed in 8 places, and all were fenced for deer protection.
   - Some maintenance has been done annually (mowing), but no irrigation was provided.
   - Survival after three years was 26/75 in the mowed plots, and 13/25 in the plot which was not mowed.
2. Barkhamsted, CT, Nepaug forest (MBC Water Co. land), 2014 seed

- 50 back-crossed, timber-type chestnut trees planted in 2015 in a clear-cut, with tree shelters

3. Harwinton, CT, Roraback Wildlife Management Area, 2014 seed
    - 50 hybrid, orchard type chestnut trees planted in 2015 around the edge of two corn fields with tree shelters

Gall wasp study.

1. Commercial chestnut trees 'Colossal' and 'Lockwood' (both gall wasp susceptible) were crossed with *C. henryi* (gall wasp resistant) in 2011, and the seedling trees planted 2012, along with 7 *C. henryi* trees from Georgia (Callaway seed) and 5 *C. henryi* from Schumacher Seed Co. None of the trees from Schumacher seed in this planting or other plantings appear to be pure *C. henryi*.
2. All of the hybrids of commercial trees had galls in 2015, but outgrew the damage.
3. The 'Lockwood' x *C. henryi* trees had fewer galls than the 'Colossal' x *C. henryi* trees.
4. None of the *C. henryi* trees are big enough yet to evaluate.

Nutrients in chestnuts. The object of this study is to see whether the nutrients present in chestnuts from commercial cultivars can be improved by using known, selected pollinizers. Open pollinated nuts from 2013 were compared with hand-pollinated nuts from 2014. Nuts were bulked, freeze dried, peeled, and analyzed by a commercial laboratory.

% dry weight

Tree/cross	Protein	Fat	Oleic acid	Linoleic acid	Linolenic acid
'Colossal' open pollinated	5.6	3.5	2.4	0.5	0.08
'Colossal' x 'Lockwood'	5.3	2.5	2.8	3.9	0.4
'Lockwood' o.p.	8.2	6.4	0.4	0.3	0.06
'Lockwood' x *C. ozarkensis* OK	11.5	3.8	3.3	3.9	0.5
'Lockwood' x *C. ozarkensis* AR	10.0	2.8	0.8	1.1	0.2
C. ozarkensis OK o.p.	8.8	12.3	6.6	2.9	0.3
C. ozarkensis OK x 'Lockwood'	8.2	10.7	4.3	2.4	0.4
C. ozarkensis OK x *C. ozarkensis* AR	10.1	22.5	4.8	2.5	0.4
C. ozarkensis AR o.p.	9.8	11.0	5.6	2.8	0.2
C. ozarkensis AR x 'Lockwood'	7.6	11.3	3.0	4.2	0.5

Tests will be repeated for several years.

Mike Marshall, Shippensburg University

Sensitivity testing of *C. parasitica*. Marshall was curious about how *C. parasitica* would respond to exogenous materials (chemicals) to which it is exposed in culture. Marshall was not looking for any particular type of response, but would expect the results of his manipulations to fall into two categories: positive (where some of the fungus is enhanced); or negative (where

some aspect is reduced or impeded). A methodology must be developed first; hopefully, one that is simple. The process then must be refined.

Marshall opted to test human pharmaceuticals for several reasons:

- They are designed specifically to alter cell physiology in some regard.
- Eukaryotic cells have a lot of physiology in common, regardless of phylogenetic diversity. This likelihood is increased as "relatedness" increases.
- This is certainly true of fungal and mammalian cells, hence the difficulties inherent in dealing with systemic mycoses in immune-compromised individuals.
- Most pharmaceuticals have a well-known mode of actions.

The methodology for the tests were as follows:

- Pour plates with glucose/yeast extract medium (20 ml/plate) or PDA (Difco).
- Inoculate small agar plug of Ep155 (5 mm from plate wall) and allow to incubate @ 21°C for 3 d in constant light.
- Apply test substance in a standard measured amount (3 mg) in standard location (5 mm from plate wall across from inoculum plug) and re-incubate under same conditions for an additional 7 d.
- Examine plates for effect. If no effects are observed, culture is discarded. If there is an effect noticed, retest substance over a range of concentrations to better define dosage/response relationship.

Most of the antifungals tested allowed *C. parasitica* to grow at least as well as controls. The antifungals, Amphotericin B and Itraconazole inhibited growth in all trials as did Naproxen sodium. Surprisingly, the Naproxen sodium produced greater inhibition than even Itraconazole and Amphotericin B, widely used antifungal medications. Other results included:

- Ethidium Bromide inhibited growth in PDA trials but enhanced growth in the 2X GYE medium.
- Aspirin inhibited growth in PDA but enhance growth in GYE trials.
- Methylene Blue inhibited growth in the 2X PDA but enhanced growth in all trials except 10X PDA combination.
- Furosemide enhanced growth in all trials except the 10X PDA combination.
- Glucotrol (glipizide) enhanced growth in the 10X GYE and both 2X treatments.
- Oxycodone enhanced growth in the 10X and both GYE trials and the 2X PDA trials.
- Ventolin (salbuterol) enhanced growth in both GYE trials.
- Enoxaparin enhanced growth in the 2X GYE trial.
- No dramatic differences were seen at 7 d in sporulation or pigmentation with any test substance.

Given the large number of materials that could potentially be tested, it would be helpful if the process could be done faster and more efficiently with less agar, space and time invested. Multiwell plates were used to solve the above issue. A 6-well plate with 4 ml agar/well gave the same depth of agar as commonly used in 9 cm petri plates. Marshall tested 6-well plates and cut down the total time by one day (to six days). He used three days of preliminary growth followed by 3 days after application. Growth was at 21° C in constant light. Nine replicates were used per *C. parasitica* isolate (using Ep 155 and Ep 146).

In reference to the materials tested, Marshall assumed that the dosage commonly used in humans would give a suitable concentration for testing against *C. parasitica*. He assumed also that the medications are commonly used to produce the desired effect in the mythical 70 kg adult. Allowing for the percentage of body mass accounted for by bone, Marshall chose a ratio to provide approximately 10X the human dose opting for 35 µl, the volume used in each well. Some of the materials are not very water soluble (partially due to fillers) and it required constant suspension of material.

Using the 6-well plates, Naproxen was still the most inhibitory compound. Many factors were at play (solubility and pH). Using NSAID ibuprofen as an example, it is a weak acid. With a pKA of 4.5, ibuprofen can be absorbed easily in the stomach but poorly in the small intestine. Most –azoles are a mainstay of antifungal pharmacology as they inhibit ergosterol production, needed for fungal membranes. Other materials tested, Meloxicam and Rofecoxib, inhibit Cox2 enzymes. All NSAID are analgesics because they inhibit prostaglandins from aracadonic acid. Cox1 enzymes are constitutive while Cox2 are inducible. There might be materials that are preferentially Cox1 inhibitors.

The NSAIDS specifics are interesting, but the larger issue is that Marshall believes there is value in this sort of exploration.

Hill Craddock, University of Tennessee, Chattanooga

The Chattanooga Report. Craddock is working with the TN TACF chapter, doing backcross breeding, selecting for blight resistance. Their orchards have been screened for blight resistance using the Meadowview model. The orchards have been rogued and they are now conducting intercrossing.

Craddock planted a Chinese chestnut in 1980 in Bloomfield, IN from seed obtained from Earl Douglass in Red Creek, NY. Craddock visited the tree in 2015 and he showed a photograph of the tree.

Seed orchards in TN are planted in stages. There are nine replications for the orchard and Craddock is looking for additional sites for planting. At this point, he has adequate seeds, but not adequate sites. He uses direct seed and transplants; there is good success in both. The seeds come from third and fourth backcross trees that have been rogued. They use open pollination when they can but they also do some controlled pollination.

In a photo from 2010, he showed nearly 100% mortality from PRR. Japanese X American hybrid was the only tree not killed. This apparently resistance hybrid can be used to backcross to Americans or to selected B_4s which gives an American type tree that has already been selected for blight resistance.

Craddock is using pollen (from 5-6 TN F_1 hybrids from a symptomatic orchards) in conjunction with the Carolina's TACF chapter. Trees are in the ground but they have not been subjected to PRR yet. During the summer of 2015, graduate student Taylor Perkins was hosted by Steve Jeffers at Clemson University and employed by Tatyana Zhebentyayeva to learn *Phytophthora* techniques. Perkins then isolated *Phytophthora* from soil samples and prepared inoculum for their own tub trials. They are using a modified Joe James system, utilizing 65-gallon horticulture pots. Kiddie pools were placed under the pots to collect the effluent as they are treating the water rather than let it flow onto the ground to kill the pathogen after it flows

through the tubs. They used chicken wire mesh to help control chipmunks. Craddock converted this experiment it into an undergraduate classroom exercise and students helped evaluate seedlings.

Korean chestnut. Years ago, Craddock reported on cultivars from Korea (Chinese X Japanese). They were selected for blight and gall wasp resistance. Craddock used these trees but he eventually lost all his grafts; however, before they were all lost, he was able to use them in breeding. One selection, 'Daebo', is now bearing nuts and he is evaluating them for fruit quality.

M. Taylor Perkins, University of Tennessee, Chattanooga

Revisiting the phylogeny and biogeography of North American *Castanea*: chloroplast and nuclear DNA sequences as indicators of cryptic diversity in the Southeast. Perkins is a MS student with Hill Craddock. He is continuing work that was started in Craddock's lab ten years ago. Trees in northwest GA/middle TN were morphologically intermediate between American chestnut and Allegheny chinkquapin. Individuals near the edge of the species' range and outliers may contain rare alleles, which are important targets for conservation. American chestnut Lincoln County 1, a highland rim chestnut from middle TN, was shown to have chloroplast haplotype P1, generally associated with Allegheny chinquapin.

Perkins collected voucher specimens and tissue samples from chinquapins at a site a few hundred yards from the Atlantic Ocean in early May. This represents the first collection of Allegheny chinquapin from the Atlantic Coastal Plain that has been made for any study on the phylogeography or population genetics of chinquapin or American chestnut—a region that authors one hundred years ago said harbored high species diversity and morphological variation in the genus.

Observations:

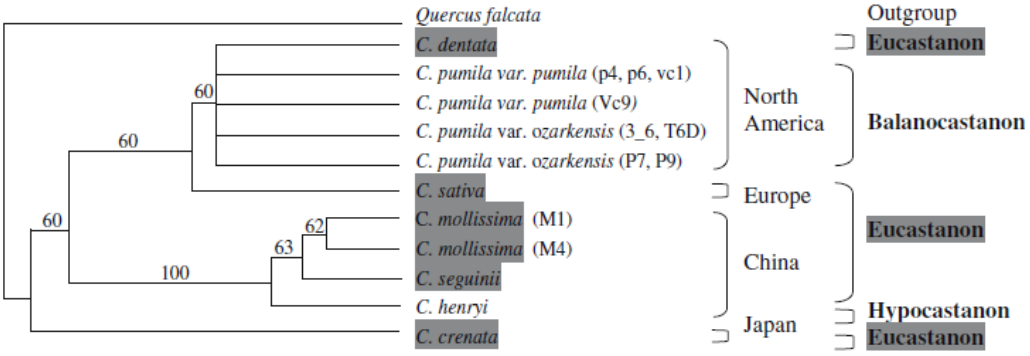
- Recent studies indicate highest genetic diversity of *C. dentata* is in the Southeastern US (Dane and Sisco 2014).
- Correspondence between chloroplast (cp) haplotypes and morphological variation is evident (Binkley 2008, Shaw et al. 2012).
- There is perfect correlation between cp haplotypes and cytoplasmic male sterility (CMS) when *C. dentata* is used as the seed parent in crosses with Asian *Castanea* spp. (Sisco et al. 2014).
- Some sites (northwestern GA, northern AL) have been documented where species identification is complicated by morphological intermediacy (Binkley 2008, Shaw et al. 2012).
- Many areas with interesting morphotypes have not been studied using molecular methods.
- Current taxonomy may not represent actual species diversity in North American chinquapins.

The Allegheny chinquapin was first described by Linnaeus in 1753; he classified it as *Fagus pumila*. Since then, 28 new names and combinations have been proposed for *Castanea pumila.* Some of the formerly recognized NA *Castanea* (chestnuts and chinquapins included):

- *Castanea ashei* (Sudworth) Ashe

- *Castanea alnifolia* Nuttall
- *Castanea alnifolia* var. *floridana* Sargent
- *Castanea alabamensis* Ashe
- *Castanea paucispina* Ashe
- *Castanea vulgaris* Lamarck
- *Castanea* x *neglecta* Dode
- *Castanea vesca* var. *americana* (Michaux) Alph. de Candolle

The last monographic revision of chinquapin in NA was published by George Johnson in 1985. He concluded that all chinquapins were one species. This was one of the last big studies prior to the use of molecular techniques. In 2006, one of the first phylogenies using chloroplast DNA was published by Lang, Dane and Kubisiak as shown below.



They concluded that sections Eucastanon and Balancastanon were not good representations of the evolution of the species. In 2007, they expanded their sequencing efforts using more regions of the chloroplast. Their conclusion was that Allegheny chinquapin is more closely related to American chestnut than Ozark chinquapin. They also surmised that the NA clade diverged from European chestnut about 39M years ago.

Kubisiak and Roberds (2003, 2006) did population genetics of American chestnut and found that:

- No disjunct regional pattern of variation exists.
- Highest levels of gene diversity and rare alleles were found in most southwestern sites.
- 95% of genetic diversity in species can be found by sampling within one population, using ssr markers.
- *C. dentata* from Tennessee, Alabama, and Mississippi were not included in final analyses (due to deletions in *trnT-trnL* that were also found in chinquapin).

Meghan Binkley (2008) sequenced trnV-ndhC (the intergenic spacer) and found that most of the American chestnut in the middle and northern part of the range has the D haplotype. The P haplotype, normally found in Allegheny chinquapin in the southern Appalachians was found in American chestnut in northern VA, middle TN and northern AL. The M haplotype was found throughout the southeast; this contains a mix of American chestnut and Allegheny chinquapin. The O haplotype found in Ozark chinquapin was also detected in northern FL and southwestern VA.

Perkins posed problems and questions.

- How many *Castanea* species are in North America?
- Where are the centers of genetic diversity for *Castanea* in North America?

- What is the extent of interspecific hybridization and introgression between *C. dentata* and *C. pumila?*
- Are different noncoding chloroplast DNA haplotypes and nuclear genotypes correlated with unique Coastal Plain morphologies?

To answer these questions, Perkins is sampling 10 individuals per site across the distribution of American chestnut and Allegheny chinquapin. In sites where there is sympatry, both American chestnut and Allegheny chinquapin, he is sampling 20 individuals. Plant height, habit, surrounding species, sun exposure, fire regime, GPS coordinates and a photo are recorded for each individual. He still needs samples from the Gulf Coast of MS and areas of LA and eastern TX.

Perkins has sampled 295 naturally occurring individuals this year from 30 sites across the range.

- Include specimens from herbaria (Botanical Research Institute of Texas, Clemson, University of North Carolina-Chapel Hill, Vanderbilt, UTC)
- *C. ozarkensis* and *C. pumila* collected from Ozarks with Jesse Harris and Dr. Joey Shaw (UTC), and Theo Witsell (AR State Botanist).
- 233 specimens from Binkley, Shaw, and Craddock's previous study.
- Additional samples of *C. dentata* used in *Phytophthora* resistance mapping, genome sequencing, and breeding work sent by Dr. Tatyana Zhebentyayeva and Dr. Paul Sisco
- In total, about 700 samples from across eastern North America.

His molecular methods are:

- DNA extraction
    - Modified CTAB protocol (Zhebentyayeva, personal communication) and DNeasy plant mini kit (Qiagen, Valencia, California, USA)
    - DNA was extracted from South Carolina samples with Dr. Tatyana Zhebentyayeva at Clemson University
    - Molecular markers and PCR
    - plastome regions used by Binkley (2008), Shaw et al. (2012), and Sisco et al. (2014): *trnV-ndhC*, *rpl16, trnS-trnG*, *rpl32-trnL*, *atpI-atpH*, and *psbA-trnH*
    - Nuclear markers:
        - *G3PDH*
        - Nuclear ribosomal *ITS* is commonly used for plant phylogenies, but may not be informative enough
        - Genotyping-by-sequencing
        - Many new EST-SSRs from Santos et al. (2015) are transferrable across *Castanea* spp. worldwide
- Quantify the degree of incomplete lineage sorting
    - Methods provided by Cummings et al. (2008) and Joly et al. (2009)

Why study both American chestnut and Allegheny chinquapin?

- To document biodiversity while we have the opportunity.
- Some morphological variation in both species may be explained by hybridization.
- If supported by the evidence, gene flow among the species should be documented and preserved.
- They are affected by many of the same exotic invasive pests and pathogens.

- Variation in the reproductive biology of North American *C. dentata* is correlated with chloroplast haplotypes shared among the species (Sisco et al. 2014).
- Knowledge of the genetic structure of both species, obtained with the same methods, can inform restoration and conservation of both species.

Andrew Jarosz, Michigan State University

Status of Asian chestnut gall wasp in MI. Asian chestnut gall wasp (ACGW), *Dryocosmus kuriphilus* was found in Michigan in June 2015 despite a quarantine banning nursery stock from states with ACGW. It was found on Chinese chestnut trees at 10 sites in southwestern Michigan. The presence of new and old galls at multiple sites indicated it has been present for more than two years. The insect was diagnosed by the MSU Plant Diagnostic Laboratory as well as by Mursel Catal in Fulbright's lab using PCR primers. PCR also verified the presence of the parasitoid *Torymus* spp. in some galls. Universal primers (ITS2-F/ITS2-R Yara) were used to amplify DNA of *T. sinensis* and *D. kuriphilus* from gall samples collected from Michigan orchards. Trapping studies revealed that the adults began emerging from the galls in late June/early July and abruptly ended emerging at the end of July.

Michigan might be the first state where the ACGW finds more European X Japanese hybrid germplasm than American chestnut or Chinese chestnut trees. Growers do have Chinese chestnut seedlings and cultivars growing, and there are thousands of American chestnuts, but Michigan orchards also are composed of all the germplasm listed in the table below. This diversity may provide insights as to how the ACGW will infest trees and orchards when more than one type of chestnut is present. European studies indicate that the European X Japanese hybrid cultivar 'Bouche de Betizac' is immune to infestation and the European X Japanese hybrid cultivar 'Colossal' is susceptible but infestations were considered minimal. So far, Chinese chestnut germplasm has been very susceptible, as has 'Labor Day' a Japanese chestnut. Growers who have planted a diversity of cultivars of European X Japanese hybrids will help inform the industry if diversity in orchards impacts the success of ACGW populations. How will cultivars in Michigan react to ACGW? Currently a large infested commercial orchard composed of Chinese chestnut seedling trees (Dunstan Hybrids) and the European X Japanese hybrid cultivar 'Colossal' is being monitored to assess the impact of infestation on both types of germplasm. So far, only the Chinese chestnut trees have shown the presence of galls, but infestation is still early. Michigan has the opportunity to test many cultivars of European X Japanese hybrids to determine susceptibility/tolerance. Growers that planted the European X Japanese hybrid cultivar 'Bouche de Betizac' should be pleased with the level of resistance to ACGW.

Commercial chestnut orchards. In 2014, the 2012 USDA Ag Survey was published. This is a survey of American agriculture taken every 5 years. Michigan still leads all other states in terms of the number of chestnut growers (over 100) and total acreage (over 800 acres planted to orchard chestnut). Production is not surveyed. However, the 32 growers comprising the state's chestnut cooperative Chestnut Growers, Inc. reported production in 2014 at 88,000 pounds. Every pound of CGI chestnut was sold to the domestic chestnut supply chain. Adding production from non-members of the chestnut cooperative places chestnut production well over 50 tons. This level of production and its high quality was surprising after the record cold winter of 2013-14. Unfortunately, winter will definitely impact the 2015 commercial chestnut

production as another record setting winter (2014-15) seriously impacted Michigan's European X Japanese hybrids as well as some Chinese chestnut trees. Trees were still recovering from the effects of winter at pollination time in Michigan. Perhaps the most important discovery found after winter damage was assessed was the high level of cold resistance found in the European X Japanese hybrid cultivars 'Marigoule', 'Marsol' and 'Maraval'. These trees were selected for blight tolerance, Phytophthora root rot resistance, ability to root, but not for cold tolerance. They are clearly Michigan's most cold tolerant cultivars.

In addition, chestnut blight has not been shown to kill the above three cultivars. *Cryphonectria parasitica* will grow in and on the bark, but it does not appear to kill the cambium or trees. It will grow on susceptible rootstock and kill rootstock, but not has not killed the cultivars. Observations suggest that blight will not kill susceptible rootstock when 'Marigoule' is grafted to it. Experiments are being set up to explore this observation.

A quick guide to cultivar characteristics found in Michigan orchards edited in 2015

Cultivar name	Presumptive germplasm	Yield + best	Nut Quality + best	Blight Sensitivity S=susceptible Tol=tolerant R=resistant	Gall wasp sensitivity	Winter/Frost sensitivity + good – poor	Root rot sensitivity
'Colossal'	ExJ	++++	++++	S	ND	++	ND
'Nevada'	ExJ	Poor	Bad	S	ND	–	ND
'Okei'	J x chinq	Bad	Bad	ND	ND	–	ND
'Bouche de Betizac'	ExJ	+++	++++	Tol	R	++	ND
'Precoce Migoule'	ExJ	++	+++	S	ND	++	ND
'Marsol'	ExJ	+++	+++	Tol	ND	+++	R
'Maraval'	ExJ	+++	++++	Tol	ND	+++	R
'Marigoule'	ExJ	+++	++++	Tol	S	++++	R
'Labor Day'	J or Korean	++	++	R	S	++	ND
'Benton Harbor'	Chinese	+++	+++	R	S	++	R

'Nevada' and 'Okei' were planted as pollinizers for 'Colossal' and are no longer recommended for planting in Michigan.

Blight: Tol = tolerant, C.p. grows but does not kill; R = no natural establishment of cankers; natural establishment of cankers leading to branch and stem death

Asian gall wasp: R = resistant; ND not determined in Michigan and not aware of any reports

Winter/Frost: – = will die in severe winters; + = not damaged during sever winters; may still be damaged in spring frosts

Michigan chestnut census. The 20th census of six American chestnut populations was completed in August 2015. Two recovering populations, County Line and Roscommon, continue to exhibit strong tree survivorship and growth. Recruitment in 2015 was disappointingly low given the heavy seed crop of 2014. Trees at the Frankfort, Leelanau, Missaukee sites all continue to decline due to the ravages of chestnut blight. No seed

production has occurred at the two Missaukee sites since 2012. Progress was made on the theoretical model that will evaluate the potential for secondary invaders to help manage chestnut blight. The first iteration of the model was published in 2015 (Eager et al. 2015).

Lynne Rieske-Kinney, University of Kentucky

Evaluating fire as a disturbance agent. There are different prescribed fire regimes being employed at Mammoth Cave to manipulate vegetation. The park is evaluating fire as a disturbance agent; this is a project in which she has been involved for 5-6 years. This study is being conducted to enhance the prey-base for forest dwelling bats. This is becoming increasingly complex due to white-nose syndrome. Mammoth Cave is the largest cave structure in the world. It is very important because it is a migratory flyway. There is a host of people working on this project from bat experts, entomologists and those who specialize in remote sensing who are using LIDAR (light detection and ranging), a method that uses light in the form of a pulsed laser to measure ranges (variable distances) to the Earth. Last year, they received extended funding from the Joint Fire Science Project to include seedlings in the prescribed burns. The goal is to look at interactions between fire and herbivory on seedling fitness (white oak, red maple and chestnut). The fire regimes were:

- Burn frequency: 1x, 2x, multi-year
- Time since burn: 2 yr, 4 yr, >6 yr

Seedling treatments:

- Fence + insecticide to exclude all herbivory
- Fencing to exclude mammalian behavior
- Insecticide to exclude arthropod herbivory
- No fencing

Seedling survival and growth will be evaluated. There are no results yet, as the seedlings were just established.

Granulate ambrosia beetle (*Xylosandrus crassiusculus*). This is an Asian species. The taxonomy and the ecology are both interesting with respect to this beetle. Ambrosia beetles are obligate symbionts. This beetle flies in the spring after three consecutive days >70°F.They feed on a fungal symbiont and rapid tree death often results from ambrosia beetle attack. The beetles house fungi in mycangia, specialized structures adapted for the transport of symbiotic fungi (usually in spore form). The beetles do not consume wood; they simply use the tree to farm the fungus. The beetles have several different fungi and many of the fungi are pathogenic. Chestnut is particularly attractive to ambrosia beetles, possibly because of the thin bark. To evaluate ambrosia beetles management, Rieske-Kinney used a push-pull strategy, a practice used mostly in agricultural systems. A repellent was used in the middle of a desired commodity and with the intent to drive the insects elsewhere. This was a paired study in which one pair received no repellent. The repellent tested was vebanone, commonly used for this insect. The attractant was ethanol/canoptheran. The push-pull system failed to work; the insects were not manipulated. There were some interesting results that occurred, however. Ninety percent of ambrosia beetles that were recovered were non-native: *X. crassiusculus* from ~70% of trap catches; *Euwallaceae validus (*the polyphagus shot-hole borer*)* 10%; and *Xyloborinus saxesenii,*

<10%. The only native species captured comprised less than 5% of all beetles. The conclusions of this small study (one season at three sites in KY) were:

- Non-native bark beetles are well established.
- Semiochemicals (verbanon, ethanol/canoptheran) work beyond the granulate ambrosia beetle.
- Push-pull strategy needs more work.

Asian chestnut gall wasp. This is an extension of Ignazio Graziosi's work on fungal lesions on galls formed by ACGW. Ignzaio performed a series of elegant experiments where he isolated the fungus, performed Koch' Postulates and he was able to infect galls with the fungus. He showed that fungal infection caused 100% gall mortality and <1% parasitoid mortality. The fungus was identified as *Colletotrichum acutatum* species complex. This fungus causes anthracnose on many plants; it also caused blossom end rot of chestnut, a problem for chestnut growers. There are strains in this disease complex that are pathogenic on plants and others that are pathogenic on the ACGW galls. The Northern Nut Growers are interested in this fungal species complex and they provided Rieske-Kinney with a small grant to look at fungal samples from blossom end rot and infected galls. She is isolating and extracting DNA and sequencing. She is comparing plant pathogenic vs entomopathogenic strains to see if there any difference in enzymatic activity—are there some specific to gall wasp.

The timing of the infection where mortality is seen. Mortality does not occur to the gall wasp larva; mortality occurs after the pupal stage when it is non-feeding. The fungi penetrate the exoskeleton of the insect.

Leila Pinchot, USDA- Forest Service

Six year results from American chestnut reintroduction trials: three plantings in the Southern Region of the USDA-Forest Service—Stacy Clark, USDA-FS and Scott Schlarbaum, University of Tennessee, Knoxville**.** Several years ago, Stacy Clark took the most resistant B_3F_3 lines available at that time and planted them in several forest settings in the southern Appalachians. The trees were planted in shelterwood cuts to look at tree growth and blight resistance. Survival at three sites for six-year-old trees was:

VA planting 68%
TN planting 68%
NC planting 77%

All the backcross trees (B_3F_3, $B_2F_3$and B_1F_3) were all significantly shorter than American chestnut but taller than Chinese chestnut, after six years. Clark used a 1-4 scale (1=best resistance to 4=worst resistance) and found at two of the three locations (VA and TN), the B_3F_3 trees were no different in blight resistance than the Chinese chestnut. At the NC location, the B_3F_3trees were worse than Chinese, probably because of a poor performing family that lowered the generational mean. Trees at the TN have not been challenged yet by chestnut blight.

Asiatic oak weevil defoliation—Ashley, Case (M.S. Student) and Scott Schlarbaum, University of Tennessee, Knoxville. The Asiatic oak weevil, *Cyrtepistomus casteneus*, has been identified as a common defoliator of chestnut seedlings. A study was initiated in eastern TN

around the perimeter of the Watuaga northern red oak orchard (near Johnson City, TN) with two primary objectives:

1. Compare the effect of three herbicide treatments on (1) defoliation severity and (2) growth of B_3F_3 chestnut and northern red oak seedlings
2. Monitor the timing and emergence of adult *Cyrtepistomus casteneus*.

Four hundred thirty 0-1 year-old seedlings (comprised of American chestnut, chestnut hybrids and northern red oak) were planted in May 2013. Four insecticides (imidacloprid, acephate, dinotefuran and a water control) were applied in June 2013 via a soil drench. Defoliation ratings were used to evaluate the efficacy periodically through the 2013 and 2014 growing season. Growth rate (RCD-root collar diameter) and height were measured yearly. Conical wire mesh insect traps were established to monitor emergence of the adult Asiatic oak weevils. There were no strong correlations between two year growth and percent defoliation implying the defoliation levels that were observed did not strongly impact early growth in the first two years. *Cyrtepistomus castaneus* emergence began in early May, peaked in late July through mid-August, and ended by late November in 2013 and 2014. The lower number of *C. castaneus* collected in 2014 may be due to cyclical emergence patterns of insects; however, further research would need to be done to confirm this. Results show defoliation to be relatively low, below 15% in 2013 and 2014 in the mid growing season and 20% in the late growing season in 2014. Mean percent defoliation ratings did not differ among American and hybrid chestnut or northern red oak seedlings. In most months, seedlings treated with dinotefuran or imidacloprid had the least amount of defoliation. Results suggest dinotefuran should be used when rapid defense is needed, but imidacloprid is recommended for long-term protection in controlling harmful defoliating insect pests. Acephate is not recommended, as it provided no discernable protection with the low level of defoliation that was observed.

American chestnut cooperators foundation report—Scott Schlarbaum, University of Tennessee, Knoxville. The 2015-2016 activities include, within the same family:

- Measure growth and assess blight resistance
- Remove dead, non-sprouting trees and prune surviving trees to facilitate mowing
- Graft scions from the best trees onto sprout

Schlarbaum has been interested in seedling quality of oak and chestnut. He has noticed that within a seed lot (when planting bare-root seedlings), there is a lot of variability in seedling height and diameter. There is a lack of uniformity within and between genetic families when using high quality seedlings. Schlarbaum is interested in developing form-classes with the hope of matching the form-class to a planting site. Each seedling is measured for height, root collar diameter and top diameter. Schlarbaum developed four form classes, based on measurement of over 11,000 northern red oak seedlings. For oak, taller, larger seedlings tend to do better in open areas with more light. Smaller chestnut seedlings (3' as opposed to 4'-5' tall) tend to do better in mid-story removal areas. Data from Leila Pinchot's Connecticut Ag. Exp. Station chestnuts planted at Vallonia State Nursery (Indiana) are as follows (from the 2015 crop):

- 30% short-thin
- 21% tall-thick
- 27% tall-thin
- 19% short-thick

- 3% cull

The cull rate was set at 2" as they found anything smaller was not worth planting.

Nurseries sell seedlings; some like TN nurseries sort seedlings into premium class and others. Even the premium class has a lot of variation. Landowners of bottomland sites may not want small-diameter seedlings but those that are thick and tall. Schlarbaum and Arnold Saxon, statistician at UT, used a macro to generate 5, 4 and 3 form-class scenarios with statistical analysis to detect difference among the classes.

Schlarbaum plans to lift ~1,000 seedlings from the East TN State Nursery, and have nurserymen visually sort by form class. Schlarbaum knows the nurserymen can sort based on 3 form classes; he is not sure about 4 or 5 form classes. Schlarbaum will then take the seedlings to his facility and measure them to see how well the nurserymen sorted by form class. If this works, it may be possible to put form classes into nursery catalogs. This issue will be discussed with field foresters.

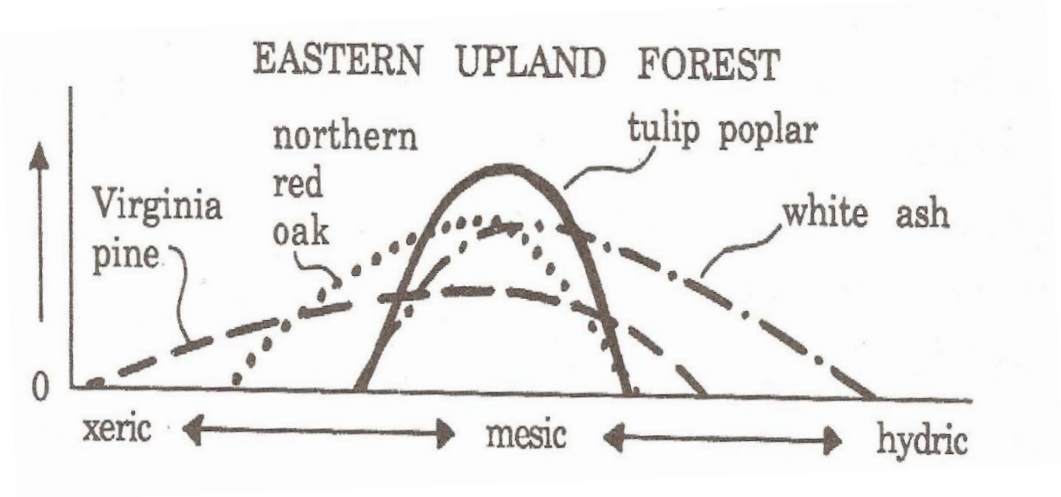
Impact of site quality on chestnut establishment success and long-term blight resistance.

The objectives of this study were:

- Evaluate the relationship between site quality and long-term hybrid chestnut growth
- Evaluate effect of site quality on blight resistance of chestnut hybrids
- Evaluate the usefulness of the Integrated Moisture Index to help select appropriate sites for chestnut reintroduction

The study was just implemented in April 2015, so she presented mostly methods.

In relation to objective 1, Gary Griffin did a study of American sprout clumps on xeric and mesic sited and found that sprout clumps do best on mesic sites when competition was controlled. Chuck Rhoades did a study in 2009 to look at site quality and silvicultural treatments and found that, after two years, seedlings did better on mesic sites. Pinchot showed the following graph to illustrate relative competitiveness among a few tree species (with competitive ability on the Y axis).



Most species grow best in mesic sites without competition. Tulip poplar cannot grow well if it doesn't have adequate soil nutrients moisture while red oak has the ability to grow in more xeric sites. That is not to say that red oak doesn't grow well in mesic sites but in many areas it cannot compete with tulip poplar so oak is pushed to more xeric sites. Chestnut, based on

older literature, grew well in mesic sites but it was more abundant on xeric sites. With respect to objective 2, she is interested in long-term dynamics.

For objective 3, Pinchot is utilizing some of the plots her colleague, Alex Royo (USFS, Pennsylvania) has established for deer browsing and foliage availability on advanced generations. Royo has 25 study sites in north central/eastern PA and all are fenced. The first question was which sites to use and how to categorize site quality. She used Integrated Moisture Index (IMI), developed by Iverson, Scott, Date and Prasad, to evaluate the usefulness of IMI to select appropriate sites for reintroducing chestnut. The IMI index is calculated using GIS layers and soils survey data as follows:

- USGS DEM Layer
    - Hillshade (solar radiation potential—40% (aspect and slope)
    - Flow accumulation of water downslope—30% (where on the slope)
    - Curvature of the landscape—10% (on a ridge or valley)
- Soil Survey
    - Total available water capacity of soil—20%

Using these four parameters, a metric was developed to categorize sites. Matt Peters, a GIS specialist developed a 1-M IMI for the 25 sites and categorized sites as mesic (IMI index 47), xeric (IMI index 31) or intermediate (IMI index 40). Based on these data, Pinchot chose 15 sites. She planted large chestnut seedlings (~4' tall) lifted from the Vallonia nursery. Pinchot planted American chestnut from the Maryland TACF chapter, Chinese chestnut from the Forrest-Keeling nursery and backcross seedlings (B_3F_2, $B_2F_1xB_3F_1$ and $B_2F_1xB_3F_1$) from S. Anaganostakis. In total, 540 seedlings were planted in April 2015 using an incomplete block design (36 trees/site) on a 12'x30' spacing. The data collection is:

- Survival
- Growth
- Competition (density of stems in a 2.5m radius)
- Canopy cover (hemispherical photos)
- Foliar soluble ions, polyamines, amino acids and chlorophyll
- Mycorrhizal associations
- Blight canker development
- *Phytophthora cinnamomi* presence

After one growing season, seedlings put on the most growth in the intermediate sites. The reason may be that on xeric sites there is less competition but also less nutrients. Mesic sites have more nutrition available but also more competition.

Gary Micsky, Penn State Cooperative Extension, Educator/PA-TACF Volunteer

Program: Leadership and Volunteer Development; Natural Resource and Environmental Management (submitted report)

Extension and Outreach Activities at Penn State. *Outreach Goal:* To develop and utilize a network of trained volunteers who can be informed and mobilized electronically to assist in multiple chestnut restoration activities.

Objectives:

I. To develop and evaluate blight resistant chestnut trees for food and fiber through traditional and molecular technologies that incorporate knowledge of the chestnut genome
II. 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
III. To develop and utilize a network of trained volunteers who can be informed and mobilized electronically to assist in multiple chestnut restoration activities

Program: Leadership and Volunteer Development; Natural Resource and Environmental Management

NE 1333 participants and TACF are valued and effective partners in my natural resources extension education programming.

NE 1333 and TACF personnel and resources have been critical to the success in expanding outreach to new audiences and have enhanced the quality of existing extension programming.

Methods:

- Training workshops and field experience
- Extension newsletters, press releases, woodland owner association newsletters
- Grower/Site evaluations
- Pest Surveys

Evaluation Process:

- Number of 2015 demonstration orchards established (N=1)
- Number of 2015 on-site test plantings established (N=19)
- Volunteers trained to identify reproductive structures and prepare for eventual controlled pollination (N=3)
- Number of volunteers trained in 2015 (N=57)
- Volunteer hours reported (*to be completed in October2015*)
- Volunteers requesting to join chestnut list serves (N=14)
- Chestnut vigor/survival on Site Assessment Plots

Volunteer Role:

- Tree ID, pollination, record keeping, culture& aftercare, program delivery
- Host research/demonstration plots
- Collect/supply genetic material
- Assist in TACF and other research activities as needed
- Advisory Committees
- Volunteer Recruitment

Volunteer Recruitment, Development, and Utilization (Objective III)

10.9.14 – 10.17.14 Open pollinated chestnut harvest & processing (10 volunteers, 26 volunteer hours)

10.14.14 "American Chestnut Science and Volunteers Making a Difference", Sharon, PA (23 participants)

02.24.15 Boy Scouts of America Demonstration Orchard planning (3 participants)

03.01.15 Materials preparation: 2015 Chestnut Schools (7 volunteers, 20 volunteer hours)

03. 14.15 "American Chestnut Site Selection and Aftercare Workshop", Mercer, PA (2 participants)
03.28.15 PA/NJ TACF Spring Meeting, Harrisburg, PA, "Best Management Practices for Growing Chestnut" (78 participants)
04.09.15 Boy Scout Demonstration Orchard site evaluation (Objectives I& II) (3 participants)
04.13.15 Mercer Garden Club "American Chestnut Restoration" presentation (22 participants)
04.27.15 "American Chestnut Site Selection and Aftercare Workshop" Mercer, PA (57 participants)
05 05.15 Demonstration Planting, Brandy Springs Park, Mercer, PA (2 participants, 7 volunteer hours)
07.14.15 Orchard Inspection, Freeman Tree Farm, Clarion County PA (3 participants) (Objectives I & II)
July 2015 photos of 2011 Freeman Tree Farm TACF Restoration Tree Planting
07.21.15 Demonstration Orchard Inspection, Lake Erie Grape Research Station Erie County, PA (5 participants) (Objectives I, II, & III)

Volunteer Recruitment, Development, and Utilization continued
08.14.15 Penn State Ag Progress Days
Conduct tours of PA-TACF/PSU breeding orchards and staff exhibit) 45 contacts

Identifying Potential Sites/Growers (Objective III)
Participants at 2015 "Grower Schools" were provided with 10 open pollinated seed and asked to provide baseline data regarding their success or failure in growing chestnut seedlings on their site. 1000 open pollinated seed were distributed to 28 individuals. Follow-up surveys utilizing the Chestnut Chatter listserv will be sent out in late September 2015.

Survey will be used to determine: 1) grower commitment; 2) site suitability for future plantings. Baseline data will include: % seed surviving, height of seedlings, weed and pest controls, tree protection, and problems encountered as of September 2015.

Burnham Tree Farm, East Finley, PA hosts several TACF plantings established in 2011, 2012, and 2013 including a small planting of BC3F3 seed.
Orchard inspection/evaluation scheduled for September 16, 2015.

US Army Corp of Engineers - Shenango Lake Demonstration Orchards established in 2013 and 2014 continue to grow and provide future value for educational programs and seed production.
Orchard inspection/evaluation will be scheduled for early October 2015.

Outreach Efforts (Objectives I, II, & III)
"American Chestnut Restoration" continues as a State-wide Program for Penn State Extension presented by the Renewable Natural Resources Team

"Chestnut Chatter" *an Extension mailing list developed in 2008 and adapted to a Penn State listserv in 2009 accommodates the need to quickly notify approximately 157 trained volunteers of program activities such as: pollination schedules, orchard plantings, harvest dates, and other labor intensive activities. (Objective III.)*
The **Winter 2015** edition of the Penn State Extension newsletter ***"The Woodlander"*** informed over 1184 subscribers throughout western PA and eastern OH about the research of NE1333 scientists Dr. William Powell and Dr. Charles Maynard at the State University of New York College of Environmental Science and Forestry (SUNY-ESF). Readers were able to explore the progress the SUNY team has made in developing a blight-resistant strain of the American chestnut tree on their project website at: http://www.esf.edu/chestnut/

The **Fall 2015** edition of the Penn State Extension newsletter **"The Woodlander"** informs and recruits potential participants for the October 2015 TACF Annual Meeting and Schatz Colloquium to be held at Penn State University. In addition, this meeting was featured in both the Spring 2015 and Fall 2015 issues of the Mercer County Woodland Owners Association newsletter "**The MCWOA News"** reaching 116 subscribers.

Business Meeting

NE-1333 is in the third year of the current 5-year project (Oct 2013-Oct 2018). Members need to be thinking who might spearhead the project renewal in 2017, as many members of the group are nearing retirement age. William MacDonald wondered if the project members would continue to meet in the absence of a formal project. Lynne Rieske-Kinney stated that she felt that project needs the structure of the regional project.

Sandra Anaganostakis indicated that she retired in August 2014. There is no current search for a replacement for her position. Sandra has all the USDA files that document every importation of chestnuts from all over the world. She has other documents that list where in the US chestnuts were sent. She would like to see these documents preserved along with her culture collection of *C. parasitica*. At this point, Sandra is still working but there will come a time when she is no longer living or working in Connecticut and she is worried about the fate of the documents/isolates. Also, CAES has all *Castanea* spp.—along with 'Mahogany', 'Graves', 'Sleeping Giant', 'Clapper'. All the trees are numbered so they are a great resource.

Bill Powell, SUNY-ESF will assume duties as the chair in 2016; he will host the meeting in New York. Hill Craddock, UT-Chattanooga, agreed to be chair-elect and host the meeting in 2017.

Respectfully submitted,
Mark Double
West Virginia University
November 2015

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Fagaceae Genetics Project

Sandra Anagnostakis
The Connecticut Agricultural Experiment Station
P.O. Box 1106, New Haven, CT 06504

2006

- Crossed 'Mahogany' X 'Nanking' pollen (*Castanea mollissima* X *C. mollissima*)
  - WL R1T15, PI# 70315 X Greg Miller pollen, PI# 108552
  - 277 nuts sent to F. V. Hebard, Meadowview, VA
  - Some seedlings returned (bare-root) in 2008, but none survived transplanting
- Crossed Spring Lot R4T52 X SpL R4T31
  - (*C. mollissima* X *C. dentata*) X (*C. mollissima* X *C. dentata*)
  - ('Mahogany' X Roxbury, CT #1) X ('Mahogany' X Roxbury, CT #4)
  - 74 nuts sent to F. V. Hebard, Meadowview, VA

2007

- Crossed 'Mahogany' X 'Nanking' pollen (as above)
  - 304 nuts planted in the greenhouse, CAES
  - seedlings tagged/numbered and individual leaves sent to T. Kubisiak in Saucier, MS for DNA
  - seedlings planted at Lockwood Farm (CAES), Hamden, CT in 2008
- Crossed SpL R4T52 X SpL R4T31
  - (*C. mollissima* X *C. dentata*) X (*C. mollissima* X *C. dentata*)
  - ('Mahogany' X Roxbury, CT #1) X ('Mahogany' X Roxbury, CT #4)
  - 77 Nuts planted in the greenhouse, CAES
  - Seedlings given to F. V. Hebard, Meadowview, VA in 2008
- Crossed SpL R4T31 X SpL R4T52
  - (*C. mollissima* X *C. dentata*) X (*C. mollissima* X *C. dentata*)
  - ('Mahogany' X Roxbury, CT #4) X ('Mahogany' X Roxbury, CT #1)
  - 58 Nuts planted in the greenhouse, CAES
  - Seedlings given to F. V. Hebard, Meadowview, VA in 2008

2008

- Crossed 'Mahogany' X 'Nanking' pollen (as above)
  - 70 nuts planted in the greenhouse, CAES
  - seedlings tagged/numbered and individual leaves sent to T. Kubisiak in Saucier, MS for DNA
  - seedlings planted at Lockwood Farm (CAES), Hamden, CT in 2010
    - trees 10 ft apart in rows 10 ft apart

- Crossed SpL R4T52 X SpL R4T31
  - (*C. mollissima* X *C. dentata*) X (*C. mollissima* X *C. dentata*)
  - ('Mahogany' X Roxbury, CT #1) X ('Mahogany' X Roxbury, CT #4)
  - 1 Nut planted in the greenhouse, CAES
  - Seedling given to F. V. Hebard, Meadowview, VA in 2009
- Crossed SpL R4T31 X SpL R4T52
  - (*C. mollissima* X *C. dentata*) X (*C. mollissima* X *C. dentata*)
  - ('Mahogany' X Roxbury, CT #4) X ('Mahogany' X Roxbury, CT #1)
  - 10 Nuts planted in the greenhouse, CAES
  - Seedlings given to F. V. Hebard, Meadowview, VA in 2009

Total number of *C. mollissima* #1 X *C. mollissima* #2 seed produced: 651

Total number of (*C. mollissima* #1 X *C. dentata* #1) X (*C. mollissima* #1 X *C. dentata* #4) seed produced: 152

Total number of (*C. mollissima* #1 X *C. dentata* #4) X (*C. mollissima* #1 X *C. dentata* #1) seed produced: 68

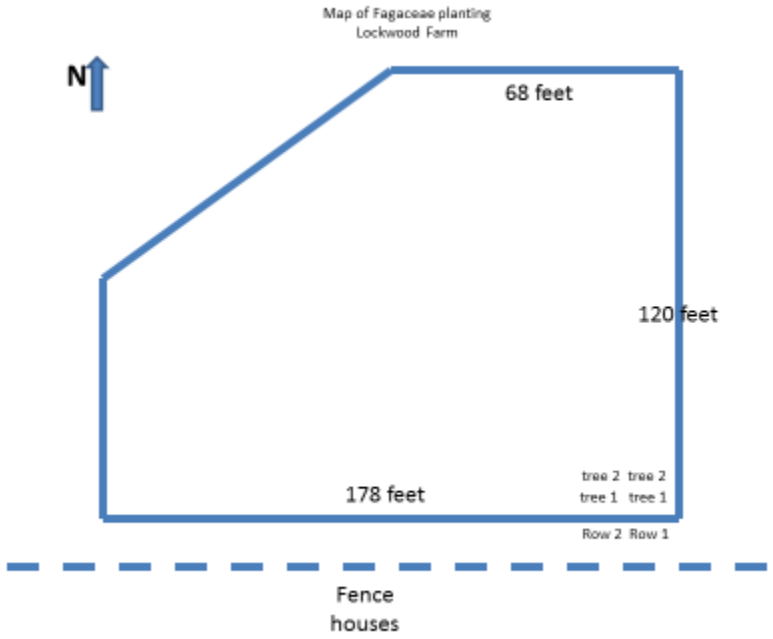
The *C. mollissima* X *C. mollissima* trees will be tended at CAES, Lockwood Farm, and available indefinitely for future genetic studies.

South fence houses planted 9 June 2010 **South West**

South	row 11	row 12	row 13	row 14	down hill
1	24829	24823	24815	24836	
2	24793	24830	24822	24837	
3	24794	24831	24817	24806	
4	24803	24832	24820	24805	
5	24842	24834	24821	24856	
6	24828	24835	24816	24852	
7	24802	24819	24810	24851	
8	24843	24814	24804	24858	
9	24844	24813	24809	24857	
10	24795	24818	24849		
11	24801	24824	24808		
12	24827	24847	24848		
13	t	24846	24850		
14	t	24825	24807		
15	t	24799	24841		
16	t	24800	24840		
17	t	24797	24838		
18	t	24796	24839		

t = unnumbered

19	t	24826	24854
20	t	24845	
21	t		
22	t		



Lockwood Farm
Fagaecae planting outlined in yellow