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

Radisson Hotel, La Crosse, WI

September 5-6, 2014

Attendance:

Canada:	Myron Smith (Carleton University, Ottawa)
Connecticut:	Sandra Anagnostakis (Connecticut Agricultural Experiment Station)
Kentucky:	Lynne Rieske Kinney, Ignazio Graziosi (University of Kentucky)
Maryland:	Donald Nuss (University of Maryland Institute of Bioscience and Biotechnology Research, Shady Grove)
Michigan:	Dennis Fulbright, Matt Kolp (Michigan State University)
New Jersey:	Brad Hillman, Administrative Advisor (Rutgers University)
New Mexico:	Angus Dawe (New Mexico State University)
New York:	Kathleen Baier, Linda McGuigan (SUNY-ESF); Steve Jakobi (Alfred Station)
North Carolina:	Jared Westbrook (TACF®, Asheville)
Pennsylvania:	Sara Fitzsimmons (TACF®, University Park); Nathaniel Cannon (Penn State)
South Carolina:	Steve Jeffers (Clemson University)
Tennessee:	Hill Craddock, Taylor Perkins (UT Chattanooga)
Vermont:	Kendra Gurney (TACF®, South Burlington)
Virginia:	Fred Hebard—chair-elect, Laura Georgi (TACF®, Meadowview)
West Virginia:	William MacDonald, Mark Double, Cameron Stauder (West Virginia University)
Wisconsin:	Anita Davelos Baines—chair, Eric Eager, Dustin Stevens, Brandon Potter, Rebekah Guthman (University of Wisconsin-La Crosse)

The meeting was called to order by Chairman Baines at 8:30 am on 5 Sept 2014 at the Radisson Hotel, La Crosse, WI. Dr. Bruce Riley, Dean of the College of Science and Health, University of Wisconsin-La Crosse provided a welcome address.

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

Kathleen Baier, SUNY-ESF

Can genetic engineering help save the American chestnut? The Powell group is using genetic engineering and biotechnology to help restore American chestnut. Hundreds of people (coordinators, visiting scientists, post docs, graduate and undergraduate students, high school students and volunteers) have contributed to this research over the past 24 years. She focused her talk on two genes: Oxalate Oxidase (OxO from wheat) and a Laccase-like protein (Lac).

OXO has a high level of enhancement (≥ Chinese chestnut). Intermediate levels of enhancement (Chinese > I > American) can be found in cisgenes, of which Lac is one. She referred to the leaf assay, an early screening assay developed by Andy Newhouse. The technique uses 6-10 leaves from fairly young tress. Several wounds are made on each leaf and a small plug of *C. parasitica* is placed on each wound. The leaves are incubated in a moist chamber (several days to a week) and then, using a software package, the area of the necrotic lesion can be ascertained. Lesions are averaged. This technique saves time as to whether trees are worth the extra effort of cloning, planting, etc.

Why are they interested in OxO? Reduced oxalic acid correlates to reduced virulence. Oxo is an enzyme that breaks down oxalic acid into hydrogen peroxide and carbon dioxide. The enzyme is not a pesticide but a plant protectant. OxO does not kill the fungus; there is no "cidal" activity, and the chance of the fungus developing resistance to OxO is reduced. On transgenic chestnut, OxO can change the fungal lifestyle form a pathogen to a saprophyte.

Field assays demonstrated enhanced blight resistance and validity of leaf assay prediction. American chestnut has a large lesion in the leaf assay and Chinese chestnut has a small lesion. One of their transgenic trees, 'Darling 4", has a lesion similar to Chinese chestnut. The leaf assay correlated well to the tree assay. 'Darling 4' has a VspB vascular promoter. 'Darling 215' and 'Darling 311" both have increased transcript levels greater than 'Darling 4'. 'Darling 215' has 40X and 'Darling 311' has 100X the Oxo level, indicating that different promoters have a big difference in OxO levels. Once again, the leaf assay correlated well with OxO levels and 215 and 311 both have smaller lesions than Chinese chestnut.

The goal is blight resistance greater than Chinese chestnut. A time-lapse video showed the results of a small stem assay over a two-week time frame. American chestnut, 'Ellis 1' died while Qing (Chinese) and 215 and 311 each showed little effect of inoculation with a virulent fungus. Baier mentioned that they often have difficulty getting the small trees to survive outdoors but they have a new graduate student who is a forester/arborist and they are hopeful that his skills will be helpful with tree acclimation/survival outdoors.

In an effort to make sure that the transgene is viable for the entire life of a tree, 3-year-old American chestnut trees were assayed for OxO expression. Stem tissue was used for qPCR and all the trees tested still maintain OxO in their genome.

Linda McGuigan does a lot of tissue culture and some of the transgenic trees do not grow as straight as seedling American chestnut. She has been able to get transgenic trees to produce pollen in the first year in a growth chamber. Pollen from transgenic trees was collected and used to pollinate flowers. Nuts were collected in the fall and the resulting seedlings were planted in the spring. The seedlings grew straight. Tissue was collected to make sure the trees were still expressing the OxO transcript in F1 seedling offspring.

A quick assay (lab or field) was developed to test for OxO persistence. Leaf disks are used to detect hydrogen peroxide. This assay takes 4 hours-to-overnight. Wheat germ is used as a positive control to make sure the assay is working correctly. The assay works well as a kit to determine which trees have the transgene.

Laccase-like gene from Chinese chestnut (cisgene). Baier showed a list of 27 "cis" candidate genes from Chinese chestnut that have been used at SUNY. They were chosen because they may have fallen into one of the resistance loci or they are more highly expressed in Chinese chestnut. Laccase is differentially expressed in American and Chinese chestnut trees. Chinese chestnut produces more laccase (100X more), whether it is inoculated or non-inoculated trees compared to American chestnut. Cisgenic trees produce laccase-like levels comparable to Chinese chestnut. However, in the leaf assay, the cisgenic trees were shown to have intermediate-size lesions. This might be due to the fact that Chinese chestnut has numerous resistance genes and the laccase-like gene may only be one of the resistance genes. Laccase-like genes enhance blight resistance, as least partly. Pyramiding the OxO with the Laccase-like gene should provide even more sustainable blight resistance. They have some events in which Oxo and Lac have been produced. They look for events that have Oxo above the 'Darling 215' level and Laccase expression that matches intermediate resistance (in leaf assays). Both of these (OxO and Laccase) should be present in shoots that they more forward. In addition, they want engineered shoots to have as many American characteristics as possible. To do this, they compared backcross trees from TACF along with a transgenic tree. Leaf tissue of each was sent to the Oak Ridge National lab for testing of metabolites. One-hundred twelve metabolites were tested. In the backcross tree, thirteen metabolites differed. In the transgenic trees, only one metabolite was different (an alpha-linoleic acid). Thus, genetic engineering produces fewer change than traditional hybrid breeding.

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 containing 150 trees each 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*. Seeds were planted annually from 2006-2011. As of July 2014, overall survival was 70%. Average diameter and height along with survival data are listed in the following table.

			Average		
Species	**Total**	**Percent Living**	**Diam. (cm)**	**Ht. (m)**	**Tallest (m)**
American	181	63%	3	3.3	7.8
B2F2	82	93%	4.2	4.4	8.9

B2F3	160	70%	2.9	3.1	7.7
B3F2	134	57%	3	3.3	8
Chinese	189	93%	4.1	4.3	8.4
European	154	44%*	3.2	3	5.5

*Low survival of Europeans is the result of poor seed quality.

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 assessed in Aug 2014 to measure canker growth 12 months after inoculation. The percentage of trees that have died from either artificial inoculation with WK-2 or from natural infections also was assessed in August 2014.

Canker Size Aug 2014	(L+W)/2 cm
American	16.2
B2s	15.9
B3s	18.6
Chinese	7.4
European	17.3

Aug 2014	% Infected Trees Dead
American	35%
B2s	16%
B3s	25%
Chinese	4%
European	27%

Hypoviruses were introduced to all naturally-occurring cankers in the three hypovirus-treated plots were treated during the 2013-2014 growing seasons by treating with a hypovirulent slurry (Euro 7, COLI, GH2 and Weekly/Ep155/pXHE7). In August 2014, 23 naturally-occurring treated cankers 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.

Laura Georgi, The American Chestnut Foundation, Meadowview

DNA markers at Meadowview. 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 F2 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 F2 population, B3 families from Pennsylvania and a very small PRR mapping population.

Updated data from the expanded F2 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 F2 population
- Screened SSRs for genotyping a Nanking backcross population

Fred Hebard, The American Chestnut Foundation, Meadowview

Overview of TACFs breeding program. The American Chestnut Foundation (TACF) is using a conventional breeding program to transfer the blight resistance of the Chinese chestnut

tree into the American chestnut tree. The only characteristic desired from the Chinese chestnut is its blight resistance, so TACF is using the backcross method to dilute out from progeny the remaining characteristics of the Chinese parent. The backcross method entails crossing the two trees to obtain a tree that is 1/2 American, 1/2 Chinese, denoted an F_1. This first hybrid is then backcrossed to American chestnut to obtain a tree that is 3/4 American, 1/4 Chinese, on average, known as the first backcross, denoted B_1. First backcrosses which manifest some blight resistance are then backcrossed again to American chestnut, to obtain trees that are 7/8 American, 1/8 Chinese, denoted B_2. A third cycle of selecting and backcrossing produces trees that are 15/16 American, 1/16 Chinese, denoted B_3. Plant breeders have found that third backcrosses are indistinguishable from the recurrent parent, in this case American chestnut, especially with co-selection for recurrent type, which TACF does. A final step is to intercross third backcrosses with each other to produce trees (B_3-F_2) that have a chance of inheriting the genes for blight resistance from both parents. Should we be able to eliminate the corresponding alleles for susceptibility to blight inherited from American ancestors, the B_3-F_2 trees will breed true for resistance, producing B_3-F_3 progeny that only have genes for blight resistance. However, regardless of whether or not all the alleles for susceptibility to blight are eliminated from the B_3-F_2 parents, evidence presented below suggests that enough genes for susceptibility to blight will be eliminated to yield nuts suitable for reforestation. If needed, further refinement of the blight resistance could occur through a program of recurrent selection conducted in the forest rather than the orchard.

Forest progeny tests. To determine whether B_3-F_3 trees can be canopy dominants and co-dominants, we are setting them out in forest progeny tests. These tests are designed to allow us to assess the long-term performance of our trees in the face of naturally occurring blight and in the face of competition from other tree species. The tests also are designed to compare the performance of different families in order to make selections at the family level.

B_3-F_3 progeny are produced in seed orchards composed of B_3-F_2 parents. In 2013, 47,200 B_3-F_3 nuts were harvested from our two seed orchards. We expect to harvest 50,000 to 100,000 nuts from those orchards in 2014, and more in the future as the parents increase in size. B_3-F_3 seedlings for progeny tests are being produced in a containerized nursery in Meadowview and in state forest nurseries in Indiana, Pennsylvania and Virginia. We have been using multiple forest nurseries for the past three years to spread the risk of crop failure.

Last winter, in 2013-2014, we transplanted B_3-F_3 seedlings into 23 forest progeny tests in GA, IN, NC, OH, KY, PA, TN, VT, VA and WV. Progeny tests were first planted on National Forests in 2009 in Regions 8 and 9, which encompass the range of American chestnut. The forest progeny tests on federal land were installed in consultation with the geneticists and silviculturists from the National Forest System and, in many cases, under the direction of or in cooperation with scientists from the Forest Service's State & Private and Research Divisions.

A manuscript entitled, "Four-year field performance of American chestnut, Chinese chestnut, and backcross generations planted in the southeastern United States," by S.L. Clark, S.E. Schlarbaum, A.M. Saxton and F.V. Hebard is in review at the journal, Forestry. It reports the earliest results from the outplantings on National Forests. Most B_3-F_3 families resemble American chestnut quite closely in metrics of growth, but quite a few resemble Chinese chestnut in time of bud break. It will be a few more years before sufficient numbers of blight infections occur to evaluate the comparative blight resistance of trees in the forest.

Orchard progeny tests. At the same time we started planting B_3-F_3 families in progeny tests in the forest, we also started progeny tests in orchard settings, where we artificially inoculate the trees with the blight fungus. The purpose of these tests is solely to evaluate blight resistance. The results of the first three years of orchard testing, in combination with results for tests of progeny from earlier generations of crossing, indicate that the average canker size on B_3-F_3 trees was comparable to that of F_1 hybrids between Chinese and American chestnut. F_1 hybrids produced in the 1940s and 1950s have been able to survive and reproduce in forest settings, which suggests our B_3-F_3 trees should too. Some individual B_3-F_3 trees test as having higher resistance than F_1 hybrids.

The blight resistance of our B_3-F_3 trees should increase beyond that of F_1 hybrids, approaching the level of resistance in Chinese chestnut, as B_3-F_2 parents that lack adequate blight resistance continue to be culled from the seed orchards producing B_3-F_3 nuts. Out of 62,343 planted B_3-F_2 nuts, currently we have culled the seed orchards to less than 11,000 trees, with a target of 700. Of those 11,000, 5,232 are selections and 6,735 were recently planted and have not been screened yet for resistance. The blight resistance of B_3-F_2 trees is assessed by the response of the trees to artificial and natural inoculation and by the performance of their progeny in orchard tests.

Phytophthora root rot incited by *P. cinnamomi*. Results from 8 years of testing breeding stock indicate that resistance to Phytophthora root rot (PRR) in our 'Graves' source of blight resistance is controlled by a single major gene. The gene does not appear to have been under selection while we were screening trees for blight resistance because PRR resistance occurred in one-half of 'Graves' second backcross families and one-quarter of third backcross families. If present in one-fourth of B_3 progeny the Hardy-Weinberg equation predicts the gene will be heterozygous in $14/64^{th}$ of B_3-F_2 progeny and homozygous in $1/64^{th}$, assuming mating is random and additional assumptions. We have observed PRR resistance at frequencies compatible with that estimate. Thus, our 'Graves' B_3-F_3 trees have resistance to both blight and PRR.

Once our 'Graves' seed orchard in Meadowview has been culled to fix blight resistance as far as possible, we plan to fix PRR resistance with two more generations of crossing. We expect the crossing will start within 2-4 years. The PRR-resistance gene should spread into all American chestnut backgrounds in 'Graves' B_3-F_3 trees, occurring in about one-fourth of the progeny. We plan to plant 46 B_3-F_3 nuts from each B_3-F_2 selection. There should be about 200 selections, so we would plant 9,200 nuts. This will give a 95% chance of obtaining at least four B_3-F_3 progeny with the gene, and four progeny would retain the inbreeding effective population size (N_ei) within 95% of starting value. After selection there would be about 800 B_3-F_3s. B_3-F_4 progeny of those B_3-F_3 selections that are homozygous for the gene should occur at frequencies exceeding one in four. Only one B_3-F_4 progeny homozygous for PRR resistance would be needed from each B_3-F_3 selection to maintain N_ei, and that one could easily be found in 10 progeny from each of the 800 B_3-F_3 selections. These B_3-F_4 trees would then be true breeding for PRR resistance in addition to the blight resistance previously incorporated into them and would constitute a seed orchard.

We believe this plan could be implemented in 10 years or less. Currently we are optimizing screening for PRR resistance in B_3-F_3 seedlings.

Research to improve selection for disease resistance. TACF makes B_3-F_2s from within a source of blight resistance to begin eliminating alleles for susceptibility to blight derived from the American chestnut recurrent parent. Complete elimination would render the B_3-F_2s true breeding for blight resistance. Direct inoculation of B_3-F_2s cannot be used to distinguish trees with one or two alleles for susceptibility from those with none; rather, some B_3-F_2s must be progeny tested, which also may not lead to elimination of all alleles for susceptibility to blight.

The amount of progeny testing required is daunting. Currently, we estimate that direct inoculation of B_3-F_2s followed by phenotypic evaluation of naturally occurring cankers will enable us to reduce their initial numbers from 27,000 to 1,000, but that reducing the 1,000 to the desired 180 will require progeny testing. The size of the task also is doubled because there currently are two sources of blight resistance. TACF will be able to accomplish the task at its professionally staffed breeding station, but we also are replicating the breeding program at most of our 16 state chapters, which are staffed by volunteers. They may have more difficulty accomplishing large progeny tests. Marker assisted selection for disease resistance offers the hope of helping select trees homozygous for blight-resistance factors, relieving some of that burden.

Currently, we are helping initiate genotyping by sequencing (GBS) using restriction site associated DNA sequencing (RAD-Seq) in a fairly small population of 'Graves' B_3-F_2s. The intent is to see whether a useful whole genome selection model (WGS) can be developed. Genome-wide association of markers developed from the sequence data (GWAS) also will be employed to see whether selection can be extended to 'Clapper' B_3-F_2s. We have been systematically collecting tissue from all of our 'Graves' B_3-F_2s since 2006, from which the DNA has been extracted and stored at the Forest Service's Southern Institute of Forest Genetics. A companion study of pathogenicity genes is underway in the blight fungus, where the pathogenicities of a set of 100 progeny have been determined and DNA extracted. The companion study may help elucidate mechanisms of resistance as well as pathogenicity.

A similar initiative for Phytophthora root rot is underway.

Aside from molecular approaches, we also have been exploring techniques for more rapid evaluation of blight resistance. The detached leaf assay does not have sufficient throughput to evaluate all of our B_3-F_2s, but may be useful in refining selections, and we are preparing to explore those uses. We also have been exploring differences between blight resistant and susceptible chestnut trees in ethylene evolution from chestnut tissue exposed to the blight fungus. Again, the technique is of low throughput but may be of use in identifying resistance in genetically transformed chestnut tissue cultures. We are exploring this.

Effective size of TACF's tree populations. TACF's current breeding populations are based on three sources of blight resistance, with two sources, 'Graves' and 'Clapper,' being the only ones from which B_3-F_3 nuts currently are being produced. 'Nanking' is the third source of resistance, but the first nuts for a 'Nanking' seed orchard were only harvested in 2013. The inbreeding effective size (N_ei) of our 'Graves' and 'Clapper' lines will be about 200 once seed orchards operated by our state chapters are producing nuts. The 'Nanking' source will increase overall N_ei to about 300.

A long-term goal of TACF's breeding program is to incorporate 20 sources of blight resistance into our breeding populations. In addition to 'Graves,' 'Clapper,' and 'Nanking,' we have about 10 sources of resistance ready to be finished by state chapters, once they have a

good start on their current seed orchards of 'Graves' and 'Clapper.' Each chapter would advance one source of blight resistance, with the final product from each source having an N_ei of 40-50. This will increase N_ei of our improved American chestnut to over 500, which should be sufficient to avoid long-term erosion of genetic diversity by drift.

Future breeding. It will not be possible to combine sources of resistance or to use recurrent selection for genotype beyond B_3-F_3 using the orchard-based methods of intense cultivation TACF has been using to present. Rather, we plan to switch to forest-based methods of cultivation for any latter stages of the breeding program, should further improvement be necessary. We would encourage natural seeding or collect seeds from forest stands to implement such an approach, with careful placement of parent trees in forest stands to promote the desired crossing. New sources of resistance would be added to existing populations as B_3-F_3s. Each source of resistance would have been backcrossed into 20 American backgrounds using orchard-based methods.

It is still unclear whether or not we will have captured enough resistance from Chinese chestnut for our B_3-F_3 trees to thrive as dominant trees in the forest, or even whether trees with blight resistance equal to that of the best Chinese chestnut trees would do so. Nor have we yet recovered enough of the American genome for our B_3-F_3 trees to constitute a viable species, but enough trees to do so are in our pipeline. However, evidence to date suggests that many of the trees currently being produced grow as well as American chestnut and have enough blight resistance to be able to survive and reproduce. In addition, resistance to PRR is present in populations derived from the 'Graves' tree. Furthermore, the trees have adequate genetic diversity to avoid immediate collapse of the population from inbreeding depression. Thus they constitute a base population for further improvement of the species.

Staffing and Facilities. The Meadowview Research Farms are staffed by a plant pathologist, a silviculturist, and a molecular geneticist, assisted by one forester, one biologist and one full-time farm hand. Additional labor is hired during the growing season. In May, 2014, over 40,000 trees were growing at 5 research farms totaling 166 acres. There are about 30 acres open and available for planting. Additional land also is made available by removing trees and replanting. At the farms, about 8,287 nuts were planted in 2014 and about 5,764 seedlings inoculated with the blight fungus to screen for blight resistance.

Pollen from advanced-generation backcross trees is shipped from the research farms to volunteers in TACF's 16 state chapters for pollination of local American chestnut trees. Inoculum is also shipped to test the blight resistance of trees in chapter orchards, and assistance provided in making crosses, growing trees and rating blight resistance. Our most advanced chapter should begin releasing B_3-F_3 seed soon. Chapters also are assisted by four TACF Regional Science Coordinators.

Nathaniel Cannon, Pennsylvania State University

Update on The Chestnut Genome Project. The sequencing of the Chinese chestnut genome was initiated in 2009 with support from The Forest Health Initiative (http://foresthealthinitiative.org/). The goal for was, and remains, to produce a reference genome sequence for *Castanea mollissima* cv Vanuxem as a platform for the identification of blight resistance genes and to conduct marker-assisted selection at the whole genome level. Participants in the project have included John Carlson (PI), Charles Addo-Quaye, Lynn Tomsho,

Daniela Drautz, Lindsay Kasson, Tyler Wagner, Nicole Zembower, Abdelali Barakat, Richard Burhans, Webb Miller, and Stephan Schuster at Penn State University, with Meg Staton, Steven Ficklin, Chis Saski, and Bert Abbott at Clemson University, Dana Nelson of the USDA Forest Service, and Fred Hebard of TACF. By 2011, the first draft of the Chinese chestnut genome was produced, consisting of 587 Mbp assembled on 51,766 scaffolds. After a second round of sequencing, a new draft genome of 724.4 Mp in 41,270 scaffolds was completed in early 2013. This represents 90% of the estimated size of the chestnut genome, in which we identified 38,268 genes supported by gene expression data. To ensure that all blight resistance genes could be identified, we also produced deep sequence of pools of BAC clones from the Chinese chestnut physical map contigs covering the three blight resistance QTL. The QTL sequences assembled into 214 scaffolds (6.8 Mb of DNA sequence) for QTL *cbr*1 on Linkage Group (LG) B, 128 scaffolds (4.1 Mb) for QTL *cbr*2 on LG F, produced 53 scaffolds (3.0 Mb) for QTL *cbr*3 on LG G. In total we identified 1952 genes in the 3 QTL, from which 15 genes were selected as high priority candidate genes for blight resistance based on defense-response gene annotations, including transcription factors, CC-NBS-LRR resistance proteins, RNA-binding protein, 14-3-3-like protein, endopeptidase, protease, disease resistance genes, glycosyltransferase, receptor-like kinase, and a histone-methyltransferase.

For the past year, Meg Staton (now at the University of Tennessee) and Ph.D. student Nathaniel Cannon at Penn State have concentrated on analyzing the current genome and QTL assemblies for publication and for use in the American chestnut breeding program. Meg Staton prepared browsers for the genome and QTL assemblies which were released at the hardwood genomics website (http://www.hardwoodgenomics.org/content/tools). The browsers are fully annotated to model plant genomes. The scaffolds, gene models, predicted transcripts and proteomes are all available to the community to download at the hardwood genomics website. A site for the genome is currently being built at the NCBI database, which is a long project in itself.

The current draft of the Chinese chestnut genome, and genome browser, were presented to the broader research community through talks and posters at the Plant & Animal Genome Conference XXII, San Diego, CA, on January 12, 2014, and at the PAG-Asia Conference in Singapore, May 19-21, 2014. Manuscripts describing the blight resistance QTL sequences and the Chinese chestnut genome are in preparation for submission in 2014. The genome manuscript will include assignments of scaffolds to linkage groups, gene family orthology groups, repetitive DNA families, statistics on genome sequence divergence among chestnut species, and the results of comparative genomic studies.

Work in the coming year will focus on establishing a solid platform from the Chinese chestnut genome for conducting genotyping-by-sequencing, transcript mapping, genome-wide association with phenotypes, and genome-wide marker selection studies. This may include an improvement to the genome assembly, leading to more complete pseudo-chromosomes. This bioinformatics research is being conducted by Nathaniel Cannon for his PhD thesis. In preparation, we have recently conducted multiplex sequencing (at 4X to 10X depth) of the genomes of several genotypes of American chestnut and Chinese chestnut, as well as parents and progeny from backcross breeding populations. Penn State will 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

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

The *Cryphonectria parasitica* genome sequencing projects: Update on JGI Genome Sequencing Project. The JGI released version V2 of the *C. parasitica* genome assembly to the public in March of 2010: (http://genome.jgi-psf.org/Crypa2/Crypa2.home.html). The Assembly continues to be a major resource for the *C. parasitica* research community. A paper describing the genome assembly has not been submitted yet.

Assembly. v2.0 (March 2010): The assembly release version 2.0 of whole genome shotgun reads was constructed with the Arachne assembler and improved with finishing reads. This release contains 26 main genome scaffolds totaling 43.9 Mb. Five scaffolds are considered complete telomere to telomere and an additional 6 have a telomere on one end. The remaining 15 scaffolds are smaller and do not contain telomeres. Roughly half of the genome is contained in 4 scaffolds all at least 5.1 Mbp in length.

Nuclear Genome Assembly:	v1.0	v2.0
Scaffold count	39	26
All contig count	536	33
Scaffold sequence bases total	43.9 Mb	43.9 Mb
Scaffolded (large) contig sequence bases total	43.6 MB	43.8 MB
Estimated % sequence bases in gaps	0.5 %	0.2 %
Scaffold N50/L50	5/4.0 Mb	4/5.1 Mb
Contig N50/L50	78/159.5 Kb	5/4.0 Mb
Number of scaffolds > 50.0 Kb:	20	13
% in scaffolds > 50.0 Kb:	99.6 %	99.6 %

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Simple and efficient recycling of fungal selectable marker genes with the Cre-*loxP* recombination system via anastomosis. (Dong-Xiu Zhang, Hsiao-Ling Lu, Xinggang Liao, Raymond J. St. Leger and Donald L. Nuss). Reverse-genetics analysis has played a significant role in advancing fungal biology, but is limited by the number of available selectable marker genes (SMGs). The Cre-*loxP* recombination system has been adapted for use in filamentous fungi to overcome this limitation. Expression of the Cre recombinase results in excision of an integrated SMG that is flanked by *loxP* sites, allowing a subsequent round of transformation

with the same SMG. However, current protocols for regulated expression or presentation of Cre require multiple time-consuming steps. During efforts to disrupt four different RNA-dependent RNA polymerase genes in a single strain of the chestnut blight fungus *Cryphonectria parasitica*, we tested whether Cre could successfully excise *loxP*-flanked SMGs when provided *in trans* via anastomosis. Stable Cre-producing donor strains were constructed by transformation of wild-type *C. parasitica* strain EP155 with the Cre- coding domain under the control of a constitutive promoter. Excision of multiple *loxP*-flanked SMGs was efficiently achieved by simply pairing the Cre-donor strain and the *loxP*-flanked SMGs-transformed recipient strain and recovering mycelia from the margin of the recipient colony near the anastomosis zone. This method was shown to be as efficient as and much less time consuming than excision by transformation-mediated expression of Cre. It also allows unlimited recycling of *loxP*-flanked SMGs and the generation of disruption mutant strains that are free of any foreign gene. The successful application of this method to *Metarhizium robertsii* suggests potential use for optimizing reverse-genetics analysis in a broad range of filamentous fungi.

Vegetative incompatibility loci with dedicated roles in allorecognition restrict mycovirus transmission in chestnut blight fungus. (Dong-Xiu Zhang, Martin J. Spiering, Angus L. Dawe and Donald L. Nuss). Vegetative incompatibility (*vic*), a form of nonself allorecognition, operates widely in filamentous fungi and restricts transmission of virulence-attenuating hypoviruses in the chestnut blight fungus *Cryphonectria parasitica*. We report here the use of a polymorphism-based comparative genomics approach to complete the molecular identification of the genetically defined *C. parasitica vic* loci with the identification of *vic1* and *vic3*. The *vic1* locus in the *C. parasitica* reference strain EP155 consists of a polymorphic HET-domain-containing 771 amino acid (aa) ORF designated *vic1a-2,* that shares 91% identity with the corresponding *vic1a-1* allele, and a small (172 aa) idiomorphic DUF1909-domain-containing ORF designated *vic1b-2* that is absent at the *vic1-1* locus. Gene disruption of either *vic1a-2* or *vic1b-2* in strain EP155 eliminated restrictions on virus transmission when paired with a *vic1* heteroallelic strain, however, only disruption of *vic1a-2* abolished the incompatible programmed cell death (PCD) reaction. The *vic3* locus of strain EP155 contains two polymorphic ORFs of 599 aa (*vic3a-1*) and 102 aa (*vic3b-1*) that shared 46% and 85% aa identity with the corresponding *vic3a-2* and *vic3b-2* alleles, respectively. Disruption of either *vic3a-1* or *vic3b-1* resulted in increased virus transmission. However, elimination of PCD required disruption of both *vic3a* and *vic3b*. Additional allelic heterogeneity included a sequence inversion and a 8.5-kb insertion containing a LTR retrotransposon sequence and an adjacent HET-domain gene at the *vic1* locus and a 7.7 kb sequence deletion associated with a nonfunctional, pseudo *vic* locus. Combined gene disruption studies formally confirmed restriction of mycovirus transmission by five *C. parasitica vic* loci and suggested dedicated roles in allorecognition. The relevance of these results to the acquisition and maintenance of *vic* genes and potential for manipulation of *vic* alleles for enhanced mycovirus transmission are discussed.

Mutagenesis of the catalytic and cleavage site residues of the hypovirus papain-like proteases p29 and p48 reveals alternative processing and contributions to optimal viral RNA accumulation (Kenneth S. Jensen and Donald L. Nuss). The positive-stranded RNA genome of the prototypic virulence-attenuating hypovirus CHV-1/EP713 contains two open reading frames (ORF), each encoding an autocatalytic papain-like leader protease. Protease p29, derived from the N-terminal portion of ORF A, functions as a suppressor of RNA silencing while protease p48,

derived from the N-terminal portion of ORF B, is required for viral RNA replication. The catalytic and cleavage site residues required for autoproteolytic processing have been functionally mapped *in vitro* for both proteases, but not confirmed in the infected fungal host. Here we report the mutagenesis of the CHV-1/EP713 infectious cDNA clone to define the requirements for p29 and p48 cleavage and the role of autoprotelysis in the context of hypovirus replication. Mutation of the catalytic cysteine and histidine residues for either p29 or p48 was tolerated, but reduced viral RNA accumulation to approximately 20-50% of the wild-type level. Mutation of the p29 catalytic residues caused an accumulation of unprocessed ORF A product p69. Surprisingly, the release of p48 from the ORF B-encoded polyprotein was not prevented by mutation of the p48 catalytic and cleavage site residues and was independent of p29. The results show that, while dispensable for hypovirus replication, the autocatalytic processing of the leader proteases p29 and p48 contributes to optimal virus RNA accumulation. The role of the predicted catalytic residues in autoproteolytic processing of p29 was confirmed in the infected host, while p48 was found to also undergo alternative processing independent of the encoded papain-like protease activities.

Characterizing the roles of *Cryphonectria parasitica* RNA-dependent RNA polymerase-like genes in antiviral defense, viral recombination and transposon transcript accumulation (Dong-Xiu Zhang, Martin J. Spiering and Donald L. Nuss). An inducible RNA silencing pathway, involving a single Dicer protein, DCL2, and a single Argonaute protein, AGL2, was recently shown to serve as an effective antiviral defense response in the chestnut blight fungus *Cryphonectria parasitica*. Eukaryotic RNA-dependent RNA polymerases (RdRPs) are frequently involved in transcriptional and posttranscriptional gene silencing and antiviral defense. We report here the identification and characterization of four RdRP genes (*rdr1-4*) in the *C. parasitica* genome. Sequence relationships with other eukaryotic RdRPs indicated that RDR1 and RDR2 were closely related to QDE-1, an RdRP involved in RNA silencing ("quelling") in *Neurospora crassa*, whereas RDR3 was more closely related to the meiotic silencing gene SAD-1 in *N. crassa*. The RdRP domain of RDR4, related to *N. crassa* RRP-3 of unknown function, was truncated and showed evidence of alternative splicing. Similar to reports for *dcl2* and *agl2*, the expression levels for *rdr3* and *rdr4* increased after hypovirus CHV-1/EP713 infection, while expression levels of *rdr1* and *rdr2* were unchanged. The virus-responsive induction patterns for *rdr3* and *rdr4* were altered in the D*dcl2* and D*agl2* stains, suggesting some level of interaction between *rdr3* and *rdr4* and the *dcl2*/*agl2* silencing pathway. Single *rdr* gene knockouts Δ*rdr1-4*, double knockouts Δ*rdr1/2*, Δ*rdr2/3*, Δ*rdr1/3*, and a triple knockout, Δ*rdr1/2/3*, were generated and evaluated for effects on fungal phenotype, the antiviral defense response, viral RNA recombination activity, and transposon expression. None of the single or multiple *rdr* knockout strains displayed any phenotypic differences from the parental strains with or without viral infection or any significant changes in viral RNA accumulation or recombination activity or transposon RNA accumulation, indicating no detectable contribution by the *C. parasitica rdr* genes to these processes.

Summary for putative vic3 locus. The *vic3* locus has been tentatively identified on the basis of two polymorphic gaps observed for EP155 (*vic3-1*) and the three *vic3-2* strains EU40, EU60, and EU55. These gaps are located on scaffold 5 and span a 7-kb-long region, >2 Mb away from the *vic1* locus. Gap 1 is 2.4 kb in size and includes the coding sequence for a hypothetical protein (599 and 614 amino acids long in *vic3-1* and *vic3-2*, respectively) in its entirety. Gap 2

consists of a short 200-bp-long region located 3.4 kb away from Gap 1 and contains the 3'-terminal end of an open reading frame for a short (102–108 amino acids in *vic3-1* and *vic3-2*, respectively), glycine-glutamine-rich protein. Disruption of the hypothetical protein in allele vic3-1 did not affect barrage formation. Studies are in progress.

One idea from this *vic* work is the possibility of knocking out individual vic genes to create a universal donor. Nuss hopes to disrupt parts of each of the vic loci to have a virus-containing strain that can be used to treat any canker, regardless of its vegetative compatibility type.

One technical problem, common to filamentous fungi, is the low number of selectable markers. With *Cryphonectria* you can use hygromycin resistance, neomycin resistance, benomyl resistance and blasticidin, so there are four markers. Using selectable markers in the field may be difficult with federal and state regulations but there is a way around this—knocking out and recycling selectable markers; this method completely eliminates the selectable markers in the final product. This can be accomplished using Cre-Lox recombination, also known as a site-specific recombinase technology. This technique is widely used to carry out deletions, insertions, translocations and inversions at specific sites in the DNA of cells. It allows the DNA modification to be targeted to a specific cell type or be triggered by a specific external stimulus. It is implemented both in eukaryotic and prokaryotic systems. The system consists of a single enzyme, Cre recombinase that recombines a pair of short target sequences called the *Lox* sequences. This system can be implemented without inserting any extra supporting proteins or sequences. The Cre enzyme and the original *Lox* site called the *loxP* sequence are derived from bacteriophage P1. The *loxP* sequence is only about 34 nucleotides long. If a hygromycin-resistance selectable marker is put in, flanked by the *loxP* sequences along with the Cre-recombinase, the selectable marker is eliminated leaving only one *loxP* sequence. The Nuss lab has developed a protocol in which the Cre recombinase is provided by anastomosis between a Cre-expressing donor strain and a recipient strain that is transformed by a *loxP*-flanked SMG. The advantages of anastomosis-mediated Cre-excision of selection marker genes (SMGs) are:

- No need to prepare spheroplasts of the *loxP*-flanked SMG transformed strain, nor for transient or stable transformation of the mutant with a Cre-expressing plasmid.
- Multiple *loxP*-SMG-*loxP*-transformed lines can be paired simultaneously with the same Cre-expressing strain, little cost is involved and considerable time savings are achieved.
- Allows unlimited recycling of *loxP*-flanked SMGs.
- Results in the generation of disruption mutant strains that are free of any foreign gene. Important feature when considering release of fungi such as *C. parasitica* and *Metarhizium* species that have been engineered for enhanced biocontrol applications.
- Successful use of this method for *M. robertsii* predicts that the method may find broad application.

Bradley Hillman, Rutgers University

Cryphonectria projects.

- Does virus infection induce activation of transposons in *C. parasitica*? (Four Class I transposons, four Class II transposons and putative *Helitrons*) in conjunction with Limie Du and Chunguang Du.

- What is the nature of the *C. parasitica* flat mutation?
- Mapping and characterization of recovering American chestnut trees in New Jersey, including canker microbiome analysis
- Using chestnut blight as a tool to introduce the breadth of research and educational opportunities at a Land Grant University (first-year undergraduate seminar course with field trips).

Transposons. Hillman has collaborated with Guohong Cai (now at USDA-ARS, Ames) on *Phytophthora* viruses and eastern filbert blight and he has spent less time recently on chestnut projects. He is hoping to begin working on chestnut projects as time allows. One question he would like to investigate—can we use *Cryphonectria* viruses as a model to ask the question whether RNA viruses may induce transposition of resident transposons. He became interested in this when Michael Milgroom began using a transposon as a fingerprint probe. Hillman indicated that he could characterize it to figure out if it was a transposon. He wanted to know if, in a haploid organism, is there any induction of transposition associated with viruses. The reason is that this is an under-investigated topic, with relatively little published data. Now we are in the position of having the genome of *Cryphonectria* characterized and we know that there are eight different types of transposons (four Class I—reverse transcribing and four Class II). One has been demonstrated to be active, and some others are predicted to be active. Limie Du, a recently hired technician in his lab, has worked on maize transposons for 10 years and thus brings a lot of molecular experience to the project. Her husband, Chunguang, is a bioinformaticist at Montclair State University in northern NJ and he also had done transposon work in maize. He has specifically worked on a new class of transposons, the helitrons, which are found in all eukaryotes and are thought to replicate by a so-called "rolling-circle" mechanism. Helitrons have been identified by bioinformatics and are poorly understood due to a lack of supporting molecular studies, but they are thought to be common and important in moving genes around. About 2% of the maize genome is comprised of helitrons. They are difficult to find because you have to write scripts specifically to find them. Chunguang is a world authority for writing scripts for helitrons. Hillman asks the questions: (1) are there helitrons in *C. parasitica*?; and, (2) can we show associated activity? There is potential, once again, to use *Cryphonectria* to investigate something of biological importance. Chunguang is going through the *C. parasitica* genome and he has identified at least one putative helitron. Working with Limie, he is asking the biological question whether this acts like a helitron and what we might do to investigate this whole class of transposons. Hillman wants to use the simple genome of *Cryphonectria* to begin to understand some of the biology of helitrons.

Flat mutation. Hillman has been working on this issue on and off for nearly 30 years. Flat mutants were responsible for getting him interested in how RNA viruses affect genomes. Now we have some of the tools to answer more questions. Flat mutants are deeply pigmented with very little aerial mycelium and the spores are produced directly on vegetative hyphae. These are produced only in culture and only in virus-infected cultures. They have been studied in CHV1-infected cultures (both French and Italian). Fulbright indicated that they have also been seen in CHV3-infected cultures. Hillman wants to study virus induction of these mutations—since he has a whole host of viruses (all in Ep155 background), we can ask the question whether the induction is specific to CHV1 or if it is broader. Mutant colonies initially contain virus, but maintenance of the virus is not required to maintain the phenotype. The

phenotype is independent of virus presence. It is a nuclear gene mutation; they do not revert. In CHV1, it is a single gene mutation; segregation in ascospores is 1:1. The flat mutant has a transposon band that was not present in the parent. Daniela Linder-Basso cloned that specific element and showed it was a new transposition event in Ep 155. Does the new transposition event follow the phenotype? Unfortunately, it does not. When you mate Ep 67 (female) with a flat mutant of the opposite mating type (male), the ascospore progeny yield a 1:1 ratio of flat:normal (all virus-free). The new transposition event did not follow the flat phenotype. After Linder-Basso graduated, the work was dropped. Now we have Illumina sequencers so the question can be asked at the genome level.

Mapping large trees. In eastern NJ, Sara Fitzsimmons introduced Hillman to some of the forests/parks in the Middletown area that contain large (>1 foot dbh) native American chestnuts. GIS specialists are mapping the large trees in the area.

Microbiome analysis. Besides *C. parasitica*, what other organisms are in a canker, either culturable or unculturable? He conceives *Castanea* microbiome as part of a teaching tool and this fall semester will use chestnut blight as a lens to introduce first-year students to the breadth of research at the New Jersey Agricultural Experiment Station of Rutgers University, including topics of invasive species, the pandemic, forest pathology, plant breeding, microbiology, genetics and genomics, etc. He can use that to connect students with faculty who are doing work in all the aforementioned areas.

Angus Dawe, New Mexico State University

Multiple pathways lead to incompatibility-mediated cell death. Genetic regulation of non-self recognition is hypothesized to be a means of limiting the spread of cytoplasmically-transmissable agents such as mycoviruses. Compatible strains will form a stable heterokaryon, while incompatible strains will seal fused compartments that subsequently undergo programmed cell death. In *Neurospora crassa,* VIB-1 has been highlighted as a transcriptional activator that is required for the expression of downstream effectors. We have explored the role of a putative *vib-1* homolog from the plant pathogen *Cryphonectria parasitica*, a model system for mycovirus-host interactions and causative agent of chestnut blight. We have found that when *Cpvib-1* was deleted from the wild-type strain EP155, the resulting phenotype included enhanced pigmentation and conidiation, similar to that reported for *N. crassa,* and pathogenicity when tested on chestnut tissue. We have further identified a role for *Cpvib-1* in mediating the incompatibility response and cell death in *C. parasitica* by testing interactions with strains that contain allelic variations at loci that control compatibility. Using a gene knockout approach, we have found that while allelic differences at *vic4* are mediated by *VIB1*, colonies with different alleles at *vic2* still maintain barrage formation, even in the absence of *VIB1*. This suggests that there is an additional mechanism for triggering incompatibility and cell death.

Investigating LysM protein function in pathogenic interactions of the chestnut pathogen *C. parasitica.* By examining genome sequence data, *C. parasitica* has been found to contain five putative proteins containing LysM motifs. These motifs have been recognized using information from the organism's genome portal. The LysM motif, found in eukaryotes and bacteria but not archaea, has been found to be implicated in many different biological

processes. Of relevance to this study is the potential to act as an effector protein, which plays a role in the virulence of certain pathogens. Chitin, a major component of the fungal cell wall, is composed of repeating subunits that can be considered a pathogen associated molecular pattern (PAMP). Many plants possess pattern recognition receptors for these PAMPs, which recruit chitinases to destroy the fungal wall, thereby combating infection. Recent findings provided evidence that LysM containing proteins in other fungal plant pathogens (e.g. *Cladosporium fulvum* and *Magnaporthe oryzae*) are secreted during the initial fungal infection of the plant. It has been determined that these LysM containing proteins are able to bind to chitin, competing with the plant's pattern recognition receptors, therefore helping to overcome the host's defense response. In an effort to identify the roles of LysM-containing proteins in the virulence of *C. parasitica*, knockouts of all five of genes have been attempted, although two (LM25 & LM83) appear to be lethal knock-outs. Of the remaining three, only one (LM47) showed significant reduction of virulence, a phenotype also coupled with a strong vegetative growth defect. Absence of LM69 did not lead to any discernible changes in phenotype from EP155 in vegetative growth or virulence. However, the last of these knockout strains, lacking LM12, did not show differences in vegetative growth but did lead to a statistically significant increase in canker size compared to EP155. This protein, when tagged with a FLAG tag allowing detection on western blots, was also found to bind chitin and was secreted into culture media. Furthermore, the mature protein was also found to be post-translationally modified by the addition of glycosyl moieties.

Myron Smith, Carleton University, Ottawa

Transcription analysis of non-self recognition associated with programmed cell death in *C. parasitica*. Smith has been working on non-self recognition systems in fungi for quite some time and several years ago he became interested *in Cryphonectria*. He provided an update of this *Cryphonectria* research, mainly done by his Ph.D. student, Anatoly Belov. There are advantages (of using *Cryphonectria*) because of the viral system, something not found in *Neurospora*, for example. Heterokaryon incompatibility can be studied in a number of ways, looking at heterokaryon formation (or lack thereof) and barrage formation. Microscopy also can be used employing stains. Hyphal contacts can be viewed under a microscope and the hyphae can be traced back from the parent strains. This can give an idea of death rate of those two contacts. He showed photos of compatible and incompatible reactions. Hyphae in compatible reactions are nice and plump while those in incompatible reactions have a series of events. First there is granualization followed by vacuolization. Finally, there is plasmolysis (shrinking of plasma membrane). Cell death is rather localized and it can by asymmetric or symmetric. Evans Blue stain can be used as it is taken up when cells are sick or dying. Cells cannot pump out the stain so they fluoresce. Interestingly, when one of the strains in a pairing contains CHV1, the amount of cell death frequency decreases significantly. This happens with either the virus or simply constructs. What really seems to be the active component of the virus is a piece of DNA that codes for p29. This suggests that the virus is manipulating the host non-self recognition system. The effect of p40 is vic3 specific while p29 works on all vic incompatible interactions. When two virulent isolates are paired, Ep155 and 74-3 (that differ at vic3), about 65-80% of the contacts have cell death in one or both of the strains. When p29 is present, cell death drops to 16-25%. (These data are based on 50 contacts per slide for 3 slides).

To determine if it is the p29 protein, some p29 stops were engineered. When p29 with the stop was introduced into Ep 155 and paired with p74-3, there was still a reduction in cell death indicating that this is an RNA effect rather than a protein effect. They had a scare in that the reactions stopped working. They found if they grew the fungus with the p29 construct for three months in continuous culture, they started to lose the suppression of cell death, so the fungus seems to be able to adapt. As a word of caution, transformants should be stored rather than maintained in continuous culture. These stops led them to hypothesize that the active inhibitor is the RNA component. Don Nuss provided Smith with Dicer knockouts (DCL2) which are deficient in RNAi. If RNAi is knocked out, there is a decrease in cell death, similar to the p29 effect.

The vic loci are all very different. They are complexes of genes. It would seem unlikely that the effect of the virus is on the vic gene, but the blockage or interference is probably at some downstream process. He is interested in dissecting the pathway whereby non-self recognition induces cell death.

To follow up on this, they conducted experiments that looked at a number programmed cell death inducers and chemicals that induce cell death. He then looked at some analysis of the transcriptome to see how the transcriptome changes with and without the virus. To get RNA from incompatible interactions, they generated a lot of colony forming units and co-inoculated onto a membrane, allowed the interactions to occur and then scraped off the tissue. RNA was then extracted. This system worked well but at what time should cells be harvested to get an optimal non-self recognition reaction? Again, Evans Blue stain was used. The number of cells that have died can be scanned and a numerical value can be gained from densitometry values. At day 1, there was not enough growth. At day 2, there was not much cell death. At day 3, there was a huge increase in the relative frequency of cell death. At day 4, they were unable to recover a great deal of RNA, so at some point after day 3 there is degradation process occurring that makes it difficult to isolate RNA. They settled on day 3 and they isolated RNA using a newly developed protocol. Illumina NextSeq 500, generated sequences of cDNAs. For each experiment (either each strain or confrontation), 50 million paired-end reads (with 151 nt at each read) were generated. In this 'counting experiment', Smith showed that there was a 10-fold increase in the mating type pheromone with an incompatible interaction (p74-3 and Ep 155) over a single isolate (p74-3). He is trying to do knockouts of the mating type pheromones to further investigate this finding. Smith showed a partial summary of the differential expression based on RNA-sequencing in the following table.

Up or Down Regulated Genes	p74-3 + Ep 155 WT	p74-3 + Ep 155p29	p74-3 + Ep155p29STOP	p74-3 + Ep 155ΔDCL-2
Up Regulated	126	147	123	211
Down Reg.	20	23	16	77
Total	146	170	139	288

The above data were from day 3 samples. The outlier is the ΔDCL-2 which is an RNAi deficient strain that also has suppression of non-self recognition cell death. There are a larger number differentially expressed genes and a disproportionate number of down-regulated genes with ΔDCL-2.

Transcriptome data was shown with just vic3 incompatibility (using Ep 155 WT and p74-3); most of the genes are not transcribed differently—just 100-200 that are.

The differential expression effect of p29 was shown with Ep155WT + p74-3 compared to EP 155p29 + p74-3. Comparing those two mixes, 101 genes were common to both. There were 37 genes unique to the p29 mix and 14 are unique to the WT mix. Somehow the 37/14 genes are differentially regulated when the p29 element is present. What is p29 doing to the transcriptome in the incompatible reaction? About 20-25% of genes in any system are unknown—even in yeast. In *Cryphonectria*, understanding is even a step removed because the functions that are annotated to a gene are based on how that gene is acting in another organism—orthologs of other organism for which the function is known. Using an enrichment analysis, differentially regulated genes were blasted to data bases to see where the matches are. They then sorted the function of the genes that were matched into gene ontology categories. Classes that were significantly enriched included: secondary metabolites and genes involved in pigmentation; sugars transport; sporulation; oxidation; cell wall biogenesis; apoptosis; alcohol dehydrogenases/oxidases; and apoptosis-inducing factors/flavine-adenine dinucleotide. The enrichment analysis was done on only 2/3 of the significantly differentially regulated gene set as 1/3 of those genes are of unknown function. Most are unique to *Cryphonectria* or just too far removed. It is this gene set that is most interesting. The trick now is to figure out the function of this 1/3—are they involved with specific actions with the viral element?

William MacDonald, West Virginia University

Introduction of hypoviruses at West Salem, Wisconsin (in cooperation with J. Cummings-Carlson, Wisconsin Department of Natural Resources; 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 2013. 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 3463 cankers have been identified in the 12 plots. Four-hundred, seventeen cankers on living trees were sampled in June 2014; 145 were newly discovered.

General observations:

- When the 12 permanent plots were established in 2001, there were 517 living stems included in the study. In 2014, 40% of the original stems in the Disease Center plots

remained alive compared to 51% in 2013. Some loss of stems may be attributed to the harsh winter of 2013-14.

- 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 80% in 2013. WS-2 and WS-3 were found at rates of 5% and 9%, respectively.

Saprophytic growth and sporulation of virulent and hypovirulent *Cryphonectria parasitica* on American chestnut (*Castanea dentata*) and scarlet oak (*Quercus coccinea*) stems. (E.S. Goddard, W.L. MacDonald and M.L. Double). The ability of virulent and hypovirulent strains of *C. parasitica* to grow and sporulate on dead American chestnut and scarlet oak (*Quercus coccinea*) stems has been investigated. Sets of stems, cut from healthy trees, were inoculated at three-month intervals (May-October) after cutting, with the goal of learning if cut stems could be colonized and serve as significant inoculum sources. Colonization and sporulation were assessed visually and by culture. Both species supported growth and sporulation with the most significant occurring on stems inoculated soon after cutting. Interestingly, with later inoculations, colonization and sporulation was greater on scarlet oak. As stems aged, other organisms became increasingly significant colonizers and undoubtedly restricted invasion of host tissue by *C. parasitica.*

Matthew Kolp, Michigan State University

Understanding the role of secondary fungi inhabiting chestnut blight cankers. There is a continuum of response by chestnut trees to *C. parasitica* from callused and superficial cankers to sunken cankers with abundant stroma. There are a lot of factors that can explain certain aspects of how we see disease in nature. Kolp used the disease triangle as a way to explain the various factors: host (species, germplasm); environment (climate, location, soil); and pathogen (virulence, genetics). Time impacts each corner of the triangle. Where do secondary, non-pathogenic fungi fit it? When chestnut blight cankers are sampled, there are other organisms besides *C. parasitica* that arise from bark plugs.

Some of his major questions include:

- Do non-girdling cankers on surviving trees harbor complex communities of fungi?
- Do secondary fungi (non *C. parasitica*) play some role in the severity of chestnut blight?
- Is so, what is/are the mechanism(s)?

Sampling work has been done at:

- West Salem (34 cankers)
- County Line, MI (29 cankers)
- Roscommon, MI (32 cankers)
- Frankfort, MI (24 cankers)
- Leelanau County, MI (16 cankers)
- Missaukee County, MI (18 cankers)
- Maryland, with Gary Carver and his “cruddy bark” trees (20 cankers)

Over 2,000 total isolates have been obtained from the above sites. Kolp has been able to identify many of the secondary organisms by genus (*Sirococcus, Paraconiothyrium, Umbelopsis,*

Penicillium and *Trichoderma*). The sampling is not balanced—some sites have more girdling cankers than non-girdling (and vice versa). Overall, he finds more secondary fungi in non-girdling cankers than in girdling cankers, across all sites.

When he looks at mechanisms, he compares inhibition as a quantifying measure. He has examined *C. parasitica* on Petri plates with other fungi, looking at inhibition. Two strains of *C. parasitica* are used (Ep 155 and LE 221) along with two types of culture media, PDA and Endothia Complete Medium (ECM). Nine different non-*C. parasitica* isolates were used to test inhibition (3 *Trichoderma* spp.; *Penicillium* sp.; 2 *Umbelopsis* spp.; 2 *Paraconiothyrium* spp.; and *Sirococcus* sp.). He has three categories of responses: (1) *C. parasitica* grows over the non-*C. parasitica* isolate; (2) the non-*C. parasitica* isolate grows over *C. parasitica*; and (3) there is a zone of inhibition between *C. parasitica* and the non-*C. parasitica* isolate. He is working on using 'Image J", a free program from NIH where he can trace the colonies at various days and compute the growth, to get precise measurements. About half of his pairings are complete. Isolate, media, isolates of non-*C. parasitica* were all significant. There also were interactions between the media and the strains of *C. parasitica* and non *C. parasitica.* LE 221 did not grow on PDA as well as it did on ECM. The three strains of *Trichoderma* were best at preventing growth of *C. parasitica* compared to other non-*C. parasitica* isolates. There was a great deal of variability in the reaction of LE 221 to the six non-*Trichoderma* isolates.

Future direction. He is taking the non-*C. parasitica* isolates from the lab and using them in the field in the vein of a mudpack technique. Agar cultures of *Trichoderma* were taped onto cankers. Areas of the canker that were not covered by *Trichoderma* had massive amounts of stroma. Metagenomics of bark microorganisms is planned, as in the current procedures, we select microorganisms that have the ability to grow on agar media. He is writing an NSF grant to explore metogenomics.

Dennis Fulbright, Michigan State University

Fulbright just returned from the Northern Nut Growers meeting in Oregon and there is chestnut blight in the northwest although it does not spread. Restriction of spread may be due to the dry conditions in the northwest.

Fulbright showed pictures of cut European chestnut stems in Italy. He has never seen 'inoculum sticks' from logs laying on the ground in Italy. Conversely, he has seen *C. parasitica* on cut stems in Turkey. He is not sure that the recovery he saw in Turkey was hypovirulence. There is a lot of germplasm diversity in Turkey due in part to the introduction of chestnut into Turkey. There were original trees in Turkey, then the Roman Legion movement of trees and finally the monk movement of trees around Europe.

Asian gall wasp was everywhere in Italy, but he has heard that it is improving, maybe due to the parasitoids that are being released. Fulbright is working with a European X Japanese hybrid, 'Bouche de Betizac' that is immune to gall wasp.

New numbers from the USDA Agricultural Census indicate that Michigan is still number one in total number of chestnut farms and acreage. Over the entire U.S. (in 2012), there were 919 farms raising chestnuts over 3,784 acres. There are nearly 700 acres of chestnuts planted in Michigan. The largest acreage (70 acres) was just planted this year.

Fulbright presented a quick guide to characteristics of cultivars found in Michigan orchards as shown in the following table.

Cultivar	Presumptive Germplasm	Yield	Nut Quality	Blight Sensitivity	Gall Wasp Sensitivity	Winter Sensitivity	Root Rot Sensitivity
'Colossal'	E X J	++++	+++	Sensitive	?	R/S	Sensitive
'Nevada'	E X J	Poor		Sensitive	?	Sensitive	Sensitive
'Okei'	J X Ching	Poor		?	?	Sensitive	Sensitive
'Bouche de Betizac'	E X J	+++	++++	Tolerant	Resistant	Resistant	Resistant
'Precoce Migoule'	E X J	++	+++	Sensitive	?	Resistant	?
'Marsol'	E X J	+++	+++	Tolerant	?	Resistant	Resistant
'Maraval'	E X J	+++	++++	Tolerant	?	Resistant	Resistant
'Marigoule'	E X J	+++	++++	Tolerant	?	Resistant	Resistant
'Labor Day'	J or Korean	++	++	Resistant	?	Resistant	?
'Benton Harbor'	Chinese	+++	+++	Resistant	?	Resistant	?

'Nevada 'and 'Okei' were planted as pollinizers for 'Colossal' but are no longer recommended for planting in Michigan. The reason 'Colossal' is resistant/sensitive to winter temperatures is that many times 'Colossal' is grafted onto rootstock of either 'Nevada' or 'Okei', both of which are sensitive to cold temperatures. Planting E X J hybrids will give a much larger yield than Chinese chestnuts, despite their winter sensitivity.

Fulbright spent two summers scratch-wounding trees in orchards. Cankers on 'Colossal' trees were treated. Treated trees do not produce as high a yield as uninfected trees but more so than dead and/or dying 'Colossal' trees. Keep trees healthy for exceptional yields and treat for moderate yields. Scratching has evolved into chain saw cuts since chain saws are so light, they are easy to use. Groups of vc types are used in the hypovirulent cocktail.

In Turkey, Fulbright saw an amazing assortment of European chestnut trees, healing and non-healing. Many trees have blight cankers but the trees survive. Turkey has a lot of diversity from areas where chestnut originated in the Caucasus Mountains, to Mediterranean dry/hot arid areas to damp/forested regions along the Black Sea. He showed photos of chestnut forests in a hot/arid area of Turkey. There is chestnut blight in the forest but there is little sign of dying trees. These trees are European grafted onto European, not Chinese. Fulbright raised the question if, in the disease triangle, is it the hot/arid temperatures that keep chestnut blight at bay? Cankers had a lot of stroma but he did not know if they were hypovirulent. 'Marigoule' hybrids (JXE) are planted in Turkey; they are blighted but do not die. Fulbright reported that there was a heavy spring frost this spring in Turkey, the first in many years. The frost impacted production of hazelnuts and chestnuts.

Fulbright closed with a photos of a 'Colossal' x Chinese rootstock that is used for grafting trials. In three instances, there were trees with dead/failed grafts, but the 'Maraval' graft on the same clonal rootstock is living. The 'Maraval' scion is alive and doing well. He was at a loss as to an explanation as to why the scion seemed to aid in survival. His feeling is that a little bit of resistance in chestnut (either from breeding or hypovirulence) goes a long way in maintaining trees. What other disease can you manipulate the host and pathogen at the same

time to change the disease triangle? We have the ability to move three angles of the disease triangle (host, pathogen and the environment) to manipulate chestnut blight.

Eric Eager, University of Wisconsin, La Crosse

Modeling and analysis of American chestnut populations subject to various stages of infection. His perspective as a mathematician is very different from all others at the meeting. He received a Ph.D. in mathematical ecology and he has been at UWL since 2012. The models given to him by Anita Baines and Andrew Jarosz served as the gateway to his understanding of chestnut blight. His motivation is:

- Chestnut blight is a classic example of how introduced pathogens can alter host population biology and overall plant community dynamics.
- The chestnut blight pathogen was introduced in the U.S. from Japan and rapidly spread throughout the range of American chestnut.
- An intracellular hyperparasite of *C. parasitica* can alter the interaction between chestnuts and the blight fungus.
- If this hypovirus creates a callus that successfully halts cankers expansion, branch longevity is increased.
- The reappearance of large reproducing chestnut trees associated with a large proportion of *C. parasitica* being parasitized by the hypovirus, is currently taken to indicate recovery of American chestnut populations, but the situation is much more complicated than that ecologically.

In a 2004 paper by Davelos and Baines, there were three categories of chestnut: disease-free; recovering; and epidemic. In that paper, they looked at eight size classes of chestnut from the smallest branches to large trees. Over time, there can be changes—trees can live and increase in size and eventually reproduce, or die and move backward in size classes. Population projection matrix models are difference equations of the form: $N_{t+1} = An_{ti}$; where n_t is a vector such that the *i*th element of the n_t is the total population of *C. dentata* in stage *i* at time t. A is the population projection matrix (a collection of an array of numbers), where the *i*, *j*th element of A is the probability of individuals of stage *i* producing individuals of stage *j* in each time step. If the matrix A is primitive (there is a positive probability that each stage can eventually contribute to every other stage) then there exists a constant c such that $n_t \approx c\lambda^t v_i$; as $t \rightarrow \infty_i$ independent of initial population n_o.

- λ is the largest eigenvalue of the projection matrix A (which is real, positive and unique).
- v is the eigenvector associated with λ (which is real, positive and unique).
- λ is referred to as the asymptotic growth rate (of finite rate of population increase in Davelos and Jarosz (2004).
- v the stable stage distribution. If one normalizes v so that its entries sum to unity, it yields the proportion of individuals in each of the population's stages as $t \rightarrow_i$. This tells you how many individuals are predicted to be small seedlings and how many are predicted to be large trees.

λ is a nice reference point. If λ is 1.01, the population is growing at 1% a year. If λ is 0.98, it is decreasing at a rate 2% a year. As t grows, λ can tell if the population grows or declines.

Davelos and Jarosx (2004) constructed separate population projection matrix models to study the dynamics of healthy, diseased and hypovirus-infected American chestnut populations. What was interesting is that they found that the λ (growth rate) was not statistically different than unity in all six (two healthy, two epidemic and two recovering) chestnut populations in the northwest Lower Peninsula of Michigan sampled.

λ_H = 1.014; λ_E = 0.995; λ_R = 0.987—all of these populations are essentially stable. However, the long-term structure of recovering populations was very similar to that of healthy populations which were both different than that of diseased populations. This suggests that recovering populations were recovering from a demographic perspective.

Eager simulated the Davelos and Jarosz models—he took the stable stage distribution for the healthy population and he simulated it for the diseased population. Not surprisingly, the population declines quite sharply when you proceed from heathy to diseased and the demography shifts as well. As time grows, all size classes of chestnut begin to decline quite substantially as you look at the population of the whole.

The population of growth rates λ are not very different when transitioning from a healthy population to a diseased population. However, diseased populations decline rather substantially per these models. As possible reason for this drastic difference, despite similar λs, is the long lifespan of the average individual within the population.

For populations where individuals have long lifespans, the eventual fates of populations with similar asymptotic growth rates λ ≈1 can be substantially different, with one population growing quire well while the other declining to 0.

- Eager studied a different model of population persistence using the basic reproduction number. To normalize for lifespan, it is often appropriate to look at the basic reproduction number *r* for each population.
- If the population projection matrix A can be decomposed into A = T + F where T is the matrix of survival and stage transition probabilities and F is the fecundity matrix, r is the leading eigenvalue of the matrix $F(1 - T)^{-1}$.
- One can interpret *r* as the average number of successful newborns produced by a typical individual in the population over the course of its life.
- For the populations modeled by Davelos and Jarosz (2004), the basic reproduction number for the healthy, diseased and recovered populations are 5.28, 0.57 and 0.19, respectively. (Even though all three had similar growth rates (λ), they reproduce at different rates). The numbers (5.28, etc.) represent the number of seedlings that will grow to reproductive size. There needs to be five times the fecundity values that we currently have to have a stable population. In the year-to-year variability there is not much difference (among chestnut populations) but in a life-span of an individual there is a large difference between healthy, diseased and recovering populations.

The basic reproduction number for healthy populations is almost 30-times larger than that of recovered populations. In fact, the basic reproduction number for diseased populations is even larger than that of the recovered population. The transient dynamics when transitioning from diseased to recovered points toward a population that, at least in the short term, shows signs of recovery. Traditional theory of population dynamics is mostly concerned with asymptotic population, but there has been some work studying transient dynamics using population inertia. The population inertia of a population initially at n_o is:

$$p_\infty = \frac{W^T n_o}{W^T v}$$

where W^T is the leading left eigenvector of the population projection matrix. This equation categorizes how a population is initially distributed. p_∞ Is a lot like growth rate, when population inertia is greater than 1, the population starting at n_o will go up in the long term and when it is less than 1 it will decline in the short term.

For the model in Davelos and Jarosz, p_∞= 073 when transitioning from healthy to diseased but p_∞ = 1.63 when transitioning from diseased to recovered. What we are seeing in the chestnut populations is backed up by these models. What we see when we look deeper is that population inertia p_∞, which is a robust measurement of transient dynamics, is high when transitioning from fungus-infected to hypovirus-infected. This shows us that hypovirus infection, while being potentially insufficient in helping chestnut populations persist in the long-run, *is able to provide the population with a boost in the short run*. This short-run boost has given us time to pursue alternative conservation measures, which our mathematical analysis are helping to guide.

Future directions include the development of *individual-based models* to track the infection levels of cells within individual cankers, communities of cankers on trees and trees within a population.

Future direction also includes the development and analysis of *linear stochastic matrix models* using the data generated by Baines and Jarosz. Finally, the more thorough analysis will include the development and analysis of *a stochastic, density-dependent disease structured integral projection model* using the aforementioned data, which is currently ongoing.

<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

Steve Jeffers, Clemson University

***Phytophthora cinnamomi* and ink disease on hybrid American chestnut.** Decline of American chestnut was caused by:

Phytophthora root rot, PRR (=ink disease)

- Caused by *Phytophthora cinnamomi*
    - Introduced into the USA in late 1700s or early 1800s
- Trees in the southern range started dying in 1800s
- Widespread death at lower elevations in southeastern states

Chestnut blight

- Caused by *Cryphonectria parasitica*
- Introduced into the USA in early 1900s
- All but eliminated remaining chestnut trees in eastern forests.

Phytophthora cinnamomi is not a fungus. It is an Oomycete in the Kingdom Straminipila (Chromista) along with brown algae and diatoms. It is more closely related to plants than to

true fungi. *P. cinnamomi* is heterothallic with two mating types (A1 and A2). A2 predominates in nature in North America. A1 is rare, except on camellia. Oospores are rare and survival is mostly by chlamydospores. Jeffers indicated that it is difficult to get *P. cinnamomi* to produce sporangia in culture. Zentmyer feels the origin of *Phytophthora cinnamomi* is New Guinea where both A1 and A2 can be found.

Ink disease on chestnut:

- First described in Europe on *Castanea sativa*
    - Initially caused by *P. cambivora*
    - Later *P. cinnamomi* also was shown to be pathogenic and more aggressive than *P. cambivora*
    - 2001—three other species were implicated in Italy
        - *P. cactorum*
        - P*. citricola*
        - *P. gonapodyides*
- In USA, only *P. cinnamomi* has been found for more than 80 years
    - In 1932, initially thought to be *P. cambivora*
    - Later determined to be *P. cinnamomi*
- However, that may change

P. cinnamomi does not like cold temperatures for prolonged time periods. Thus, it is thought not to exist above the Mason-Dixon Line and in higher elevations. This may change with global warming.

Ink disease is caused by several species of *Phytophthora*. Currently, the focus is on finding resistance to *P. cinnamomi*. Resistance to one species may or may not provide resistance to the other species of *Phytophthora*. Eventually, we should be screening for resistance to all species.

Jeffers showed the 1945 distribution map of ink disease on American chestnut and chinkapin (Crandall, Gravatt, and Ryan, Phytopathology 35:162-180). He discussed the distribution of the two tree species relative to ink disease.

The American Chestnut Foundation:

- Founded in 1983
- Mission: "...to restore the American chestnut tree to its native range within the woodlands of the eastern U.S..." by breeding for resistance to *C. parasitica*
- 1989: initiated breeding program at Meadowview, VA using backcross method
- Chinese chestnut (*C. mollissima*) is used as a source of resistance.

Ink disease was rediscovered in the southeast. After 10-15 years, hybrid seeds were planted in the field for evaluation. In southeastern states, root rot killed a high percentage of seedlings in the first few years after out-planting. *P. cinnamomi* was confirmed as the pathogen in 2003 at the Chestnut Return Farm in Seneca, SC (farm of Dr. Joe James, a retired orthopedic surgeon). Dr. James was working on the knee of the chair of the forestry department at Clemson University and when James had dying trees, the department chair sent Jeffers out. They found about 100% recovery of *P. cinnamomi*; James lost about 75% of his chestnut hybrids. James and Jeffers have been colleagues now for more than a decade.

Ink disease gets its name from the color of the lesion that looks like India ink. There is often tiger-striping in the cankers on young trees.

Research objectives:

- Develop a system to evaluate hybrid chestnut seedlings for resistance to *P. cinnamomi*
    - Want to mimic what happens in the field
- Evaluate hybrid seedlings from families bred for blight resistance (*C. parasitica*)
    - Is resistance to *P. cinnamomi* also present?
    - Identify families with high levels of resistance, if present
- Eventually, create a resistant population of trees for future breeding efforts

The experimental design is as follows:

- Site, Chestnut Return Farm, Seneca, SC
- So far, 10 years of screening, 2004-2013. The 2014 trial is in progress
- Seed families are provided by TACF chapters and cooperators
    - Backcross hybrids—F1 to BC4 generations
    - American—susceptible control
    - Chinese—resistant control
- Replicates = blocks in 568-Liter plastic tubs
    - Seeds from each family are planted in each tub
    - Filled with Fafard 3B mix—a soilless container mix
    - The number of reps: 2 in 2004; 4-9 in 2005-2013
- Germinated seeds planted in rows by family
    - Families randomized in each replicate tub
    - Planted in early April
- Tubs are watered and fertilized as needed throughout the summer
    - Tubs have drains.
    - Hardware cloth screen is used to keep out mice
    - Seeds are lined out in rows
    - Maps are made of each tub
- Inoculum—2 isolates of *P. cinnamomi*, both originally from Chestnut Return Farm
    - Grown on sterile vermiculite moistened with V8 juice broth
- Inoculations are conducted in early July, 12-14 weeks after planting
    - 50 ml of inoculum are sprinkled in furrows between rows: furrows are 1-3 cm deep
    - Inoculum is placed in every other row
    - Plants begin dying 2-3 weeks later
- Tubs are flooded to saturation 1-2 times/season to encourage disease development
- Plants begin to show symptoms about 16 days after inoculation
- Evaluation
    - Plants are evaluated in late December or early January
    - Each plant is dug and scored individually
        - 0=healthy, no visible lesions on roots
        - 1=root rot on lateral roots
        - 2=root rot on tap root or severe lateral root necrosis

        - 3=plant dead
    - Survivors are transplanted into pots for 1 year and then moved to the orchard
        - Primarily transplanted plants are ones rated 0 and 1
        - Durable resistance in the field is evaluated

Results—in general, the screening methods have been successful. American chestnut seedlings have died while Chinese chestnut seedlings consistently are healthy. Two hundred hybrid families were evaluated between 2004-2013, from F1 to BC4 generations, both open pollinated and controlled crosses. Hybrid seedlings varied in their susceptibility. Ratings ranged from 0-3. Most were susceptible and died, but some survived with ratings of 0-1. Percent mortality (from 2004-2010) ranged from 68% to 99%. There is a lot of variation.

In summary, over 200 backcross families have been evaluated (many families are identical). Hybrid families selected for resistance to *C. parasitica* also carry resistance to *P. cinnamomi*. Genes for resistance to *P. cinnamomi* and *C. parasitica* do not appear to be linked.

SUNY transgenic plants (from Bill Powell and Chuck Maynard) also are being evaluated. Scott Merkle (University of Georgia) also has several lines that are being tested. These plants contain a synthetic anti-microbial peptide. Most of the trees that are genetically engineered for resistance to chestnut blight do not survive the *P. cinnamomi* screening. Jeffers and Merkle are trying to develop a method using callus tissue that can be used to assess susceptibility.

Jeffers is collaborating on several other projects. He is collaborating with Albert Abbott to identify genes associated with resistance to *P. cinnamomi*. He is collaborating with Stacy Clark at USDA-FS and with TACF on isolation and distribution of *Phytophthora* spp. from chestnut trees and soils in the eastern U.S. Jeffers has a new graduate student working on this project. Jeffers also is investigating variation among three species of *Phytophthora, P. cinnamomi, P. cambivora,* and *P. heveae*. In particular, *P. heveae* has been reported in the southern Appalachian Mountains but never reported as a pathogen with the exception of Rhododendron leaves. He is curious as why some *Phytophthora* species are found in nature but they do not kill plants. He speculated that maybe *P. heveae* killed chestnut early on in the epidemic but with the lack of chestnut, it is hard to detect. Jeffers also wants to study variation among isolates of *P. cinnamomi*, along with population genetics. It was reported years ago *Phytophthora* species that killed black walnut were not pathogenic on chestnut and vice versa. Jeffers is interested to see if the same holds true for American and Chinese chestnut.

Ignazio Graziosi, University of Kentucky

Asian chestnut gall wasp (ACGW) fecundity. Can modulating fecundity (egg load) increase invasiveness of ACGW (*Dryocosmus kuriphilus*)? ACGW is a global pest of chestnut. Chestnut resources are increasing in North America (American chestnut restoration programs, nut production, strip mine reclamation and plating of urban and landscape trees). ACGW is monophagous on *Castanea*; it is univoltine, parthogenetic, pro-ovigenic with a short adult life span (3 days). ACGW is pro-ovigenic (parasitoids that have a full complement of mature eggs at eclosion; the eggs were produced from nutrients obtained during larval development)—the egg load is fixed with no extra eggs and no egg resorption. ACGW produces galls on new vegetation that affects tree growth and reproduction. Adult wasps emerge in early summer; they will locate chestnut buds and oviposite in buds. Eggs hatch and newly hatched larvae remain within the buds throughout the fall and winter. The following spring, galls will appear

on new vegetation and larvae feed, pupate and emerge in early summer. Wasps will locate trees and lay eggs. ACGW is an aggressive invader that uses local prevailing winds. After the first report of gall wasp in the U.S. in Georgia in 1974, it has spread throughout the range of American chestnut. Infestation widely fluctuates. For this reason, he is interested in evaluating ACGW reproductive potential. He specific objective is to evaluate fecundity and correlate to insect size and age to understand invasiveness.

To assess the fecundity of ACGW, he determined the size and age of insects using 4 age-cohorts (0, 1, 2 and 3-days old). Length and width of each insect was measured along with the mesosomal (thoracic) and metasomal (abdominal) length and width (3 wasps/age cohort). He also measured the length of the hind femur. Insects were mounted on wax and dissected. The ovaries were extracted, stained and placed in glycerol and then teased apart. He counted the number of eggs from six wasps from each age cohort and egg metrics (length, width and volume) were measured (10 eggs/wasp and 3 wasps per age cohort). GLM was used for analysis. Significant differences were found among all parameters. Newly emerged wasps had, on average, 300 eggs. This number decreased with age. Egg volume was greatest for wasps two days after emergence. In terms of body metrics, newly emerged wasps are heavier and wider. The best parameter to predict egg load was body weight and metasomal width. General insect size is not a good predictor of egg load as newly emerged wasps carry a large egg load. He found that ACGW resorpt eggs in absence of the host. Nutrition resources are reallocated. Reproductive success translates to invasiveness. He thinks ACGW may not be strictly pro-ovigenic but may be synovigenic.

Summary:

- Invasiveness is influenced by potential fecundity.
- General insect size is not a good predictor of egg load.
- Newly emerged (0 d) wasps carry large egg loads.
- Egg load decreases with age.
- Egg volume is greatest the second day after emergence, then decreases.
- Insect weight and metosomal with can predict egg load.

Wasp/blight interaction. This work is in progress. An experiment was designed to see how interactions between two invasive organisms (ACGW and chestnut blight) impact the native tree. Greenhouse experiments were conducted with gall wasp-infested potted seedlings. He summarized an older experiment in which *Nectria* was used in conjunction with gall wasp and found that canker size was greater in the absence of gall wasp. Conversely, canker size was smaller with gall wasp. This experiment was repeated using *C. parasitica*. In the summer of 2013, galls were collected and wasps were released on caged Chinese chestnut seedlings. In spring-fall 2014 galled seedlings were categorized into low galls (1-4) and high galls (≥5). Seedlings were inoculated with a hypovirulent (Ep 60—Michigan) and a virulent (Wisc. 25-1) isolate. Lesion expansion was measured weekly. None of the seedlings died. He hopes to come up with a conclusion with the two invasives.

Sandra Anagnostakis, The Connecticut Agricultural Experiment Station

Asian chestnut gall wasp (*Dryocosmus kuriphilus*) resistance. Gall wasp has been present in CAES planting since 2011. All of the species of *Castanea* are planted on Experiment

Station land in Hamden, CT. One tree of *C. henryi* is the only mature survivor of the species and has no galls. In 2011, cultivars 'Colossal' and 'Easton' were crossed with *C. henryi* and the following spring, the resulting seedlings were planted under a heavily gall tree. In the spring of 2014, all the seedlings had galls. 'Eaton' X *C. henryi* trees had fewer galls than 'Colossal' X *C. henryi* and all the trees outgrew the galled areas by mid-summer.

Among the other chinquapins in her plantings, *C. floridana* shrubs are usually severely damaged in the winter and have no chance to exhibit gall infestation. *C. pumila* shrubs have occasional galls and *C. ozarkensis* trees from both Arkansas and Oklahoma have no galls. Crosses were made in 2013 using both AR and OK *C. ozarkensis* with trees of cultivars, 'Colossal' and 'Eaton' and in 2014 with 'Colossal' and 'Lockwood'. The resulting seedlings will be evaluated for resistance to gall wasp invasion and some crossed further to try and determine how resistance is inherited.

Nutrients in chestnuts. Tests made by Senter et al. (1994) found minor differences in fiber and carbohydrates between species of chestnuts but large differences in protein and fat. Among the fatty acids, Senter suggested that oleic acid was perhaps the most important in contributing improved flavor to the nuts. Anagnostakis and Devin (1999) reported that nuts from controlled hand-pollinations had differences in the amount of protein and total fat in the nuts and subsequent work found differences in fatty acid amounts in nuts from the same kind of crosses.

In 2013, she collected open pollinated nuts of 'Colossal', 'Lockwood' and the two C. ozarkensis types and had the nuts tested for protein content and fatty acid contents as a base-line (grams/100 g dry weight).

Fatty Acid/Protein	'Colossal'	'Lockwood'	Ozark (AR)	Ozark (OK)
Oleic	2.42%	0.15%	5.67%	6,63%
Linoleic	0.54%	0.26%	2.87%	2.94%
Linolenic	0.08%	0.07%	0.24%	0.31%
Protein	5.54%	8.2%	9.9%	8.8%

Since previous work had shown that the male parent does influence the fatty acid and protein content of the nuts, some of the nuts from the crosses done for gall wasp studies (above) will be tested for these nutrients.

'Fagaceae Project' Chinese chestnuts planted in CT. In 2008, 253 seedlings of 'Mahogany' x 'Nanking' (PI# 70315 x PI# 108552) were planted at the CAES farm in Hamden, CT and in 2010 another 60 seedlings were planted. All of these seedlings were numbered and sampled (leaves) for DNA and the information was used in project analyses. The numbers are recorded on the planting map. She still maintains these trees and they are available for future genetic studies.

Hill Craddock, University of Tennessee, Chattanooga

The Chattanooga Report. Craddock is working with TACF and the Tennessee chapter of TACF, a very enthusiastic chapter that originated in the early 1990s. Craddock is helping the TN chapter grow advanced backcross hybrid trees. A grafting workshop was hosted by Craddock at Meadowview to show participants the finer art of grafting, using budding tape, etc. Craddock

uses several grafting techniques, bud grafts, whip and tongue, etc. Bark inlay grafts often develop normal, healthy branch collars in trees.

Germplasm from southern sources is taken to the TACF farm in Meadowview. Craddock has taken pollen to Meadowview for 10-15 years. He noted that female flowers in the south are reproductive before northern pollen is ready.

There is a good deal of cytoplasmic diversity among chestnut. F1 hybrids made in the south are sometimes male fertile. Chloroplast genotype makers are used to find males that are fertile. Cytoplasmic male sterility is found in the offspring of interspecific crosses between American and Asian chestnut trees. If one crosses an American chestnut as female by an Asian chestnut as male, the resulting F1 tree is almost always pollen sterile. If the cross is made in the other direction, with the Asian chestnut as female and the American chestnut as male, the offspring are male fertile. This is probably caused by an incompatibility between American chestnut mitochondria and Chinese chestnut nuclear genes, as is found in many other plant species. However, exceptional male-fertile F1 progeny of American (female) x Asian chestnut (male) have been found in the southern U.S. This is the result of unusual cytoplasm in American chestnut trees in the South, as evidenced by variation in chloroplast DNA. The 'D' type chloroplasts that are associated with cytoplasmic male sterility are found almost exclusively in American chestnut trees in the Appalachian Mountains and in the central and northern parts of the range (north and east of TN). The exceptional cytoplasm that result in male-fertile F1s are most common in the southern U.S. (northern Alabama, northwest GA, northeast MS, and central TN). Some of these chloroplast types are related to chloroplasts found in Allegheny chinquapin. Some trees produce astaminate catkins—they have odor but no anthers or stamens.

Craddock is producing first backcross trees, some with putative resistance to *Phytophthora*. He takes pollen from F1s and puts onto surviving chestnuts. Those trees that do not survive or have American form are rogued out. The ones that remain are really good trees. There are backcross orchards scattered across small farms in the south.

Polar vortex. Craddock has stored seedlings outdoors in pots in Chattanooga for years, but the winter of 2013-14 produced temperatures low enough that about half of his unplanted nursery stock was killed. The plants froze. The winter also was hard on trees in the field. Some trees, although they had chestnut blight, were doing well until the 2013-14 winter; many of the trees died.

Craddock recommended some chestnut cultivars for commercial nut production, based on ten years of observations in Tennessee:

- 'Shing' (large tree, most vigorous cultivar in the trial, very productive)
- 'Gideon', 'Qing', and 'Sleeping Giant' (all excellent quality nuts, medium-sized trees)
- 'Payne' (tested as Byron 3-3, large nut on a very compact tree)
- 'Marigoule' (extra-large nuts, Euro-Japanese type, great tree form)

Craddock is evaluating seedlings from controlled pollinations for their potential as nut cultivars. Some promising crosses include:

- 'Marigoule' x 'Gideon' (to combine tree form and nut size of 'Marigoule' with superior nut quality of 'Gideon')
- 0-7 (a complex American hybrid) x 'Daebo' (a Chinese x Japanese hybrid cultivar from Korea)

M. Taylor Perkins, University of Tennessee, Chattanooga

The effect of phosphite fungicides on mycorrhiza formation in a family of BC4 Chinese-American chestnut seedlings. Perkins is a MS. Student with Hill Craddock. This work is part of a senior honor project that he did as an undergraduate student.

Phytophthora cinnamomi:

- Pathogens of the *Phytophthora* genus are one of the obstacles facing chestnut restoration in North America.
- Significant losses of American chestnut and advanced backcross trees have occurred at the Catoosa, GA orchard.
- This pathogen also has been a problem in the UTC greenhouse/nursery.

One way they try to control *Phytophthora* is with phosphite. The purpose of this study was to determine the effect of phosphite fungicides on beneficial fungi, mycorrhizae. *Pisolithus tinctorius*, an ectomycorrhizal fungus (ECM), is added every year in the UTC greenhouse to aid in seedling establishment. Thus, beneficial fungi are necessary to successful chestnut seedling establishment.

Phosphite-based fungicides:

- Active ingredient: mono- and di-potassium salts of phosphorous acid.
- Marketed as phosphite systemic fungicides.
- Phosphite may act directly on *P. cinnamomi* by reducing growth.
- Phosphite also might provoke a defense response by the plant.
- The targeted pathogen is an oocmycete, but what effect could phosphite fungicides have on chestnut ectomycorrhizae?

American chestnut forms ECM with members of the Ascomycota and Basidiomycota. ECM assist with water and nutrient adsorption (especially P, which is often growth-limiting). ECM also helps with carbohydrate storage and pathogen biocontol.

Three ECM species were chosen to inoculate seedlings. Half of the group was treated with phosphite fungicide while the other half received no phosphites. The hypothesis was that chestnut seedlings, given the manufacturer recommended dose of phosphite fungicide throughout the growing season, would have fewer ECM-infected root tips. The ECM species used were: *Scleroderma geaster, Scleroderma citrinum and Pisolithus tinctorius*.

Materials and method of his study:

- Measured ECM colonization of the roots of chestnut seedlings receiving one of four types of inoculation treatment (non-inoculated control; *Scleroderma geaster; Scleroderma citrinum; and Pisolithus tinctorius*). Spider mites interfered with the *P. tinctorius* treatment resulting in no data.
- One hundred, sixty fourth backcross Chinese-American hybrid trees were used in the study.
- The experiment was divided into 8 groups determined by fungus inoculum and phosphite treatment (phosphite was added every two weeks throughout the growing season).
- ECM morphotypes were identified and ECM infection of roots was measured.

- Perkins used SPSS to perform analysis of variance (ANOVA) to determine the main and interactive effects of the manufacturer-recommended dose of phosphite (2.4 g phosphite/L) and fungal inoculants on ECM infection.

Treatments

Treatment Group	Treatment Code	Fungus Inoculations	Routine Phosphite Applications
A	Pt + P	*P. tinctorius*	Yes
B	Pt no P	*P. tinctorius*	No
C	Sg + P	*S. geaster*	Yes
D	Sg no P	*S. geaster*	No
E	Control + P	None	Yes
F	Control no P	None	No
G	Sc + P	*S. citriunum*	Yes
H	Sc no P	*S. citriunum*	No

Spores were removed from the peridioles and gleba from dried specimens and weighed. A dry suspension was made with potting medium for seedling inoculation. Phosphite was added as a soil drench.

A unique metric was used to measure ECM (the number of ectomycorrhizal second-order lateral roots per 10 cm of first-order lateral roots). Every root sampled was given a value that was averaged for every plant and treatment group. Two morphotypes were encountered. No fruiting bodes were observed so morphotypes were used (dark brown mantle, bright white mantle).

Treatment	Avg. Number Mycorh. of Both Morphotypes
Sg + P	5.91
Sg no P	8.23
Control + P	1.51
Control no P	11.81
Sc + P	5.26
Sc no P	10.11

In summary:

- Observably, chestnuts treated with phosphite exhibited fewer ECM root tips than untreated individuals in all four inoculation levels.
- This difference was significant for seedlings in the control and S. citrinum inoculum groups but not for seedlings in the *S. geaster* inoculum groups.
- These data suggest phosphite-based fungicides may impede root colonization by ECM.

Steve Jakobi, Alfred State College

Nutrient medium for sexual reproduction of *Cryphonectria parasitica*. The traditional method for producing perithecia *in vitro* takes time and scratched, autoclaved chestnut stems. Not all researchers have access to chestnut stems, so his objective was to find an artificial nutrient medium to facilitate sexual production between mating type compatible strains (Mat-1 and Mat-2) of *C. parasitica*. Jakobi used five isolates of each mating type. Prior to testing

artificial media, trials were conducted using chestnut stems with test isolates Ep 146, Ep 155, Schomberg and Bockenauer to ensure the formation of perithecia. Subsequently, the same four strains were used to evaluate all media. Only Leonian agar amended with "light" coconut milk was used to test 29 different combinations of mating type crosses.

Nutrient media investigated included:

- Potato dextrose agar (PDA) x chestnut bark extract
- PDA amended with 1 % hemp oil
- PDA amended with 0.1% hemp oil
- PDA and dialysis membrane
- PDA amended with strawberry juice
- Asthana-Hawker medium A
- Asthana-Hawker amended with 0.01% thiamin and biotin
- Filter paper yeast extract
- Corn meal agar
- Sordaria mating agar
- YEKAC yeast medium
- Leonian medium
- Leonian amended with 0.01% hemp oil
- Leonian amended with 0.1% hemp oil
- Leonian amended with 0.01% thiamin and biotin
- Leonian amended with 1% concentrated coconut milk
- Leonian amended with 0.1% concentrated coconut milk
- Leonian amended with 1% "light" coconut milk

Of the 18 nutrient preparations tested, only 4 induced the formation of perithecia:

- PDA x chestnut bark extract (18/52)*
- Leonian agar (19/53)
- Leonian agar amended with 0.01% thiamin and biotin (23/40)
- Leonian agar amended with 1% "light" coconut milk (81/143)

* number of plates with perithecia/total numbers of plates

Sexual crosses attempted with Leonian amended with 1% "light" coconut milk.

Schomberg x Bockenhauer	EP155 x 6-3-1C
Schomberg x EP155	EP155 x 5-6-1F
Bockenhauer x Schomberg	EP155 x 7-5-1C
Bockenhauer x EP146	7-5-1C x MN-1
EP146 x Bockenhauer	7-5-1C x MN-3
EP146 x EP155	7-5-1C x 6-7-1
EP146 x 6-7-1	7-5-1C x EP155
EP146 x MN-1	6-7-1 x Schomberg
EP146 x MN-3	6-7-1 x 7-5-1C
EP155 x EP146	6-7-1 x EP146
EP155 x Schomberg	6-7-1 x 6-3-1C
MN-1 x EP146	6-3-1C x 6-7-1

MN-1 x 7-5-1C 6-3-1C x MN-1
MN-1 x Schomberg 6-3-1C x EP155
MN-3 x 7-5-1C
Twenty-five of twenty-nine crosses produced perithecia. Some crosses never produced perithecia, irrespective of the media used.

To ensure that the perithecia produced viable ascospores, perithecia from several of the above crosses were examined. Perithecia were exposed in the stroma, pierced with a sterile dissecting needle and serially diluted onto PDA. Germinating ascospores were plated onto PDA and they grew to form colonies, proving that Leonian agar with coconut milk produces perithecia with viable ascospores.

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

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

- 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 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 2014 research/test orchards established (N=1)
- Number of 2014 on-site test plantings established (N= 32)
- Volunteers who learn to identify reproductive structures and correctly prepare female flowers for pollen exclusion and eventual controlled pollination (N=13)
- Number of volunteers trained in 2014 (N=79)
- Volunteer hours reported
- Volunteers requesting to join chestnut list serves (N=40)
- Chestnut vigor/survival on Site Assessment Plots

Volunteer Roles:

- Tree ID, pollination, record keeping, culture and 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 Activities

- 10.08. 13 OP Seed Harvest Collect and process open pollinated American chestnut seed for TACF use, volunteers (N=6) Volunteer Hours: >16

- 01.09.14 Meeting with National Fuel Gas and J. Burnham regarding potential funding of research/production orchard in Washington County, PA,7 participants
- 03.01.14 Materials preparation: 2014 Chestnut Schools, 7 volunteers, 20 volunteer hours.
- 03.01.14 Annual Asian Gall Wasp Survey (*in support of Dr. Lynn Riske-Kinney's research*), Volunteers: (N=2) Volunteer Hours: 3
- 03 08.14 "American Chestnut Site Selection and Aftercare Workshop" Clarion, PA, 18 participants
- 03. 14.14 "American Chestnut Science and Volunteers Making a Difference" Erie, PA, 23 participants
- 03.22.14 "American Chestnut Site Selection and Aftercare Workshop" Mercer, PA,13 participants
- 05 09.14 US Army Corp of Engineers Shenango Lake Demonstration Planting, 9 participants 27 volunteer hours
- 06.30.14 Pollination Clinic, Freeman Tree Farm, Clarion County PA, 13 participants
- 07.05.14 Prep and bag American chestnut trees for F1 production, Sandy Lake, PA, 2 participants 4 volunteer hours
- 07.10.14 Collect/Process *C. mollissima* pollen for F1 production, 2 participants 4 volunteer hours
- 07.17.14 Pollinate Sandy Lake PA American chestnut "mother trees", 2 participants 4 volunteer hours
- 07.22.14 Chestnut Update for Mercer County Woodland Owners Association, 15 participants
- 07.22.14 Interview with The Sharon Herald Newspaper – *"focus on chestnut volunteerism"*
- 07. 17.14 Pollination for F1 Seed Production, Sandy Lake, PA, 3 participants 6 volunteer hours
- 08.14.14 Penn State Ag Progress Days (Conduct tours of PA-TACF/PSU breeding orchards and staff exhibit), 38 contacts
- 08.19.14 Planning session: Boy Scouts of America Eagle Scout Project in Chestnut Restoration, 2 participants

Identifying Potential Sites/Growers

- Participants at 2014 "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 38 individuals. Follow-up surveys utilizing the Chestnut Chatter listserv will be sent out in late September 2014.
- 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 2014.
- Burnham Tree Farm, Washington County, This orchard was host to a 7.19.14 field tour led by Sara Fitzsimmons of TACF for over 100 participants attending the 2014 National

Tree Farm System Convention. Burnham Tree farm hosts several TACF plantings established in 2011, 2012, and 2013 including a small planting of BC3F3 seed.

- US Army Corp of Engineers - Shenango Lake Demonstration Orchard established on 05. 09. 2014. This orchard will complement the 2013 restoration planting and add additional educational value.

Outreach Efforts

- "American Chestnut Restoration" is officially listed as a State-wide Program for Penn State Extension 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 250 trained volunteers of program activities such as: pollination schedules, orchard plantings, harvest dates, and other labor intensive activities.
- "Chestnut Gall Wasp – Monitoring a New Threat" a Penn State Cooperative Extension fact sheet, that was developed with assistance from Dr. Lynne Rieske-Kinney, University of Kentucky and NE-1333 participant. This fact sheet was utilized again in 2014 in three American Chestnut Site Selection and Aftercare Workshops to enlist volunteers in monitoring the spread and severity of this pest in western PA and eastern OH.
- Penn State Cooperative Extension newsletter "The Woodlander" informed over 1184 subscribers throughout western PA and eastern OH of chestnut-related educational opportunities.

Business Meeting

Brad Hillman, Administrative Advisor, reminded the Chair, Anita Davelos Baines, that the 3-page summary is due 60 days from the conclusion of the meeting. NE-1333 is in the second year of a 5-year project. The current project runs from 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. This becomes relevant with regard to who benefits from USDA-approved projects—mostly people who are associated with an Experiment Station. The number of members of the group who have NIPA-approved projects is getting smaller.

There are other ways of having a USDA-approved project that are not research-related. There are coordinating committees but Experiment Station Directors do not pay salaries or travel for coordinating committees. This is simply a vehicle to get people together. It is reasonable that we could coordinate with TACF and become a subset of that group. As long as there is interest, there should be no problem getting another approval in 2018 because the breadth of the work is fantastic.

Money is allocated on an Experiment Station basis; it is not equal. The formula for funds is quite complicated. Hatch and Hatch-multi-state projects give Experiment Station Directors the possibility to pay for salaries or project funding.

Fred Hebard, TACF, will assume duties as the chair in 2015; he will host the meeting September 10-12, 2015, either at Hungry Mother State Park or at the Marion County 4-H camp near Meadowview. Bill Powell, SUNY, agreed to be chair-elect and host the meeting in Syracuse, NY in 2016.

Respectfully submitted,
Mark Double
West Virginia University
October 2014

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