NE-1033 Technical Committee Meeting
Biological Improvement of Chestnut through Technologies that Address Management of the Species, its Pathogens and Pests

Boone Tavern, Berea, KY

September 6-7, 2013

Attendance:

California:	Pam Kazmierczak, Neal Van Alfen (UC-Davis)
Connecticut:	Sandra Anagnostakis (Connecticut Agricultural Experiment Station)
Indiana:	Jeanne Romero-Severson (University of Notre Dame), Michael French (TACF®, IN)
Kentucky:	Lynne Rieske Kinney—chair, Ignazio Graziosi, Lou Shain (University of Kentucky), Lynn Garrison (TACF®, KY), Jennifer Koslow (Eastern Kentucky University), Harley Weaver
Maryland:	Donald Nuss (University of Maryland Institute of Bioscience and Biotechnology Research, Shady Grove), Sunshine Brosi, Michael McCampbell (Frostburg University)
Michigan:	Andrew Jarosz, Dennis Fulbright, Matt Kolp, Claire Moore, Josh Springer (Michigan State University)
New Mexico:	Angus Dawe, Didi Ren, Xiaoping Li, Megan McClean, Mona Pokharel, Karyn Willyerd (New Mexico State University)
New York:	Linda McGuigan, Kristen Stewart (SUNY-ESF)
North Carolina:	Bryan Burhans, Paul Sisco, (TACF®, Asheville), Russell Regnery
Pennsylvania:	Kevin Burke (University of Pennsylvania), Gary Micsky (Penn State Extension-Mercer)
South Carolina:	Tetyana Zhebentyayeva (Clemson University)
Tennessee:	Hill Craddock (UT Chattanooga), Stacy Clark (USDA-FS, Knoxville)
Virginia:	Jeff Donahue, Fred Hebard, Laura Georgi, David Bevins (TACF®, Meadowview), Matt Brinckman (TACF®, Charlottesville)
West Virginia:	William MacDonald, Mark Double (West Virginia University)
Wisconsin:	Anita Davelos Baines—chair-elect (University of Wisconsin-La Crosse)

The meeting was called to order by Chairman Rieske-Kinney at 8:00 am on 6 Sept 2013 at the Boone Tavern, Berea, KY. Dr. Chad Berry, Academic Vice President and Dean of Faculty, Berea College, provided a welcome from Berea College.

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

Tetyana Zhebentyayeva, Clemson University

Mapping resistance to root rot disease in chestnut. Before the arrival of *Cryphonectria parasitica*, the southern portion of the range of American chestnut was partially wiped out by *Phytophthora cinnamomi*. Japanese and Chinese chestnut are resistant to both *C. parasitica* and *P. cinnamomi*. Since 2004, Phytophthora root rot phenotyping (PRR-test) has been conducted at the Chestnut Return Farm in Oconee County, SC by Steve Jeffers, Joe James and Paul Sisco. Chestnut seedling are planted outside (April) in plastic tubs with a soilless mix (20 seeds/cross). Seedlings are inoculated with two *P. cinnamomi* isolates from the study site. Since 2012, Clint Neel and Paul Sisco organized an additional site for phenotyping the PRR resistance. In a TN location, they have been using a lower plant density in the plastic tubs. Infested soil in the bottom of the container serves as a 'natural' source of *P. cinnamomi*. The American and Chinese chestnut are used as controls in all experimental tubs. Preliminary test for two phenotypic classes (alive or dead) is based on the above-ground appearance in the end of vegetation season in September. Final scores are obtained by phenotyping of fully dormant plants for severity of root rot symptoms in December. A 0-3 subjective rating system is used to assess the roots (0=healthy root; 1=lesions on feeder roots; 2-lesions on tap root; and 3=dead plant).

Breeding for resistance to both pathogens simultaneously is essential before American chestnut can be restored to its full native range. Using combined genetic and genomic approaches, resistance to *C. parasitica* (*Cp*) has been mapped to three quantitative trait loci (QTLs). In addition, a marker set covering the chestnut genome has been generated for implementation in breeding for *Cp* resistance. Although *P. cinnamomi* (*Pc*) was introduced to the USA far earlier than *Cp*, an effort to breed for resistance to this pathogen has been initiated only recently. Selection of parental genotypes with a high inter-generational transmission rate of resistance to *Pc* allowed initiation of genetic studies. The initial mapping efforts with a limited number of progeny (48 individuals) were made on an interspecific BC_1 cross: AdairKY1 (American chestnut) X GL158. The *Pc* resistance in this cross was derived from Chinese chestnut, *C. mollissima* 'Nanking'. DNA was collected from healthy plants before inoculation. Based on the above-ground phenotyping, a segregation ratio was 1:1 (27 alive vs. 4 struggling/17 dead). A low density map composed of 12 linkage groups was constructed using 203 SNPs. A QTL for resistance to *Pc* was detected and mapped to linkage group E (LG_E).

Three backcross families (HB1, HB2 and MK5) from another source of resistance (*C. mollissima* 'Mahogany') were selected for map construction and association analysis with 22 markers from LG_E. Preliminary analyses confirmed the presence of a *Pc* resistance QTL on the LG_E. In 2012-2013, extended mapping populations (up to 200 individuals) representing the 'Mahogany' and 'Nanking' lineages of resistance were planted for phenotyping of *Pc* resistance and QTL mapping. Together, these materials and analyses should help resolve the location of the QTLs for resistance to *Pc* and test their co-location between two important sources of resistance in chestnut.

Future steps include:

- Increasing progeny size up to 1000 individuals in the HB2 cross ('Mahogany' background)
- New sources of resistance from *C. crenata* and *C. mollissima* ('Nanking')
- Advanced B_3F_1 plant material ('Mahogany')
- Improve reliability of the PRR test by extending the evaluation to a 3-year period
- Construct a complete genetic map for genome-wide QTL detection using genotyping-by-sequencing (HB2 cross)

Linda McGuigan, SUNY-ESF

American chestnut somatic embryo transformations—comparison of semi-solid medium method to a temporary immersion bioreactor method. She has worked with Chuck Maynard to transform American chestnut with blight resistance genes. She initially used a gene gun and obtained poor results. In 2004, an *Agrobacterium*-mediated transformation system was developed to transfer putative resistance genes into the plant's genome. She reported on a comparison of a transformation system that used either a (1) semi-solid medium or a (2) temporary immersion bioreactor system (RITA®) during the selection stage. The RITA® system is typically used to grow plant tissue by temporarily flooding the culture with a nutrient solution. To do the standard transformation, she mixed *Agrobacterium* and American chestnut somatic embryos for an hour. The embryos were then placed on a desiccation plate (a sterile Petri plate with moist filter paper) for a few days and then transferred to an *Agrobacterium*-kill (*Agro*-kill) medium containing antibiotics that kill the *Agrobacterium*. Finally, they are transferred to a selection medium, which is the same as *Agro*-kill with an additional antibiotic used to kill any non-transformed cells. She compared this method to that of the bioreactor to determine if it might help increase the number of transformants.

Methods included:

- Co-transformation
  - *Agrobacterium tumefacians* (AGL1) – pFHI-LTP2 (lipid transfer protein)
  - *Agrobacterium tumefacians* (AGL1) – pGFP (green fluorescent protein)
- Desiccation plates for two days.
- *Agro*-Kill plates for one week.
- Selection with paromomycin (par) to kill any non-transformed cells:
  - Petri dishes with semi-solid medium (84 µM par)
  - Bioreactors with liquid medium (25 µM par)
  - Bioreactors with liquid medium (75 µM par)
  - Bioreactors with liquid medium (200 µM par)
- The medium was changed every two weeks.
- After 20 weeks, tissue was tested for the two genes using multiplex PCR with two sets of primers.

The bioreactor created many more transformants than the standard procedure. The 200 µM par bioreactor treatment created 18 events with only the gene of interest compared to 0 for the semi-solid medium.

Conclusions:

- The best treatment was the bioreactors with 200 µm paromomycin (26-fold more effective than the standard semi-solid medium).
- The bioreactors allowed production of transgenic events without the gfp marker gene.
- Total time to obtain individual events:
  - Standard method: approximately 6 months
  - Bioreactor method: 2-4 months
- Additional studies continue to show higher rates of transformation.
- Getting a lot more transformation in a shorter amount of time using the bioreactor.

Kristen Stewart, SUNY-ESF

Can the FT1 gene from poplar be used for rapid cycle tree breeding in American chestnut? Key features of her study included:

- This is strictly a greenhouse study initially using leaves for expression.
- We know the FT1 gene drives other genes.
- We will need to do tests for other genes or transcription factors using other plant parts (example- AIL 1 in buds and meristem).
- Monitor *FLOWERING LOCUS T (PtFT1)* gene from Poplar (*Poplar trichocarpa) for expression using Rt-qPCR.*
- We are presently using viable pollen from plants in the high light growth chamber (no *Pt*FT1 gene present) in the field.
- This greenhouse study would need both male and female flowers to test for the presence of the FT1 gene in progeny.
- With no FT1 gene, then, and only then, could the plants be introduced into a field test site (similar to the rapid cycle crop breeding plum studies being done in WV).

Objective: second generation gene constructs and transformations:

- Production pipeline selection (culling):
  - Co-transformations selected on semi-solid medium using paromomycin and ppt, and then visually select for GFP (Lilibeth Northern)
  - PCR SE clusters to confirm presences of CG in events using UBQ11/CG primers to distinguish transgene from native gene, cull as needed (Kristen Stewart)
  - Multiply SE clusters and go through four stage shoot generation (~10 weeks). Self-culling since not all events generate shoots. (Allison Oakes)
- Production pipeline testing:
  - Single shoots are multiplied to represent one event to remove any escapes or possible mixed events. (Allison Oakes and Kristen Stewart)
  - PCR is repeated to identify escapes, which are then culled. (Kristen Stewart)
  - Representative shoots are tested by RT-qPCR method to determine relative expression levels compared to a non-transformed clonal line (Ellis 1). Expression

looks at transgene and native gene expression. (Kathleen Baier and Kristen Stewart)
    - Key flowering time regulators: Native FT, PTFT1 and AIL 1 transcription factors will be tested by RT-qPCR method to identify possible signaling pathways and the regulation of meristem activity. (Note: AIL 1 is part of a family of genes that regulate floral development.(Kristen Stewart)
- Research Objective:
    - To examine events from the clonal line Ellis 1 and the HSP driven FT1 (HSP/FT1).
    - Attempt to induce flower development with an induction temperature of 40˚C with different exposures of 30-90 minutes daily for three weeks.
    - Test at ambient temperatures (~22˚C) as temperature controls.
    - Monitor morphological changes, *PtFT1* gene expression and endogenous genes expression associated with flowering using RT-qPCR for comparisons.

Looking at flowering pathways:

- Genes of Interest:
    - FT1 is primarily involved in flowering. Analysis in poplar species have indicated that FT1 could be primarily involved in reproductive growth, while FT2 could be primarily involved in growth cessation. (Karlber et al. 2011)
    - Both FT1 and FT2 arrange the repeated cycles of vegetative and reproductive growth in woody perennial trees. (Hsu et al. 2011)
    - FT1 is primarily involved in flowering. Analysis in poplar species have indicated that FT1 could be primarily involved in reproductive growth, while FT2 could be primarily involved in growth cessation. (Karlber et al. 2011)
    - Both FT1 and FT2 arrange the repeated cycles of vegetative and reproductive growth in woody perennial trees. (Hsu et al. 2011)
    - *FLOWERING LOCUS T (Pt*FT1*)* gene from Poplar (*Poplar trichocarpa) has* been found to stimulate early and continual growth. (Srinivasan et al. 2012). The relative expression of *Pt*FT1 will be measured using leaf samples from both flowering and non-flowering plants.
    - The AIL 1 gene could be a good reporter gene to make sure the FT1 is working even before flowers are detected (AIL 1 is downstream induced by FT1 creating an expression cascade).
    - AIL 1 expression is a potential downstream target of the Short Day (SD) signal in cessation of growth and bud set in hybrid aspen and poplar. (Karlberg et al. 2011; Hsu et al. 2011).
    - Various research studies have shown that the AIL 1 gene expression is significantly higher in young dividing tissue including (seedlings, roots, and inflorescences) as compared with mature tissues (rosette leaves and stems).

We are they headed:

- Latest Trials:
    - 67 American chestnut trees
    - 4 separate transgenic events, Ellis 1 non-transgenic controls
    - Three week heat inductions
    - RNA extractions, cDNA, RT-qPCR

- Continue multiplying shoots in tissue culture (the four transgenic events and Ellis 1) as back up stock
- *FLOWERING LOCUS T (Pt*FT1*)* gene from Poplar (*Poplar trichocarpa) has* been found to stimulate early and continual growth. (Srinivasan et al. 2012). The relative expression of *Pt*FT1 will be measured using leaf samples from both flowering and non-flowering plants.

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 August 2013, overall survival was 77%, specifically:

Species	Total	Percent Living
American	181	76%
B2F2	82	94%
B2F3	160	73%
B3F2	134	69%
Chinese	189	94%
European	154	59%

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

Average diameter and height are listed in the following table.

	Average		
Species	Diam. (cm)	Ht. (Feet)	Tallest (Feet)
American	1.9	5.2	17'
B2F2	2.7	9.5	21'
B2F3	1.9	5.9	17'
B3F2	1.8	4.5	20'
Chinese	2.5	9.1	19'
European	1.7	3.6	11'

On 31 July 2013, eighty-seven trees >3 cm (15 American; 24 BF2; 13 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.

To introduce hypoviruses, naturally-occurring cankers in the three hypovirus-treated plots were treated on 5 Aug 2013 with a hypovirulent slurry (Euro 7, COLI, GH2 and

Weekly/Ep155/pXHE7). The treated cankers will be subjectively rated annually to assess growth, sporulation and host response.

Paul Sisco, The American Chestnut Foundation, Carolinas Chapter

Orchard effect on blight resistance. There is a significant interaction between orchard location and blight resistance. Two full-sib families of backcross chestnut trees from the same seed lots were planted at different locations in NC in 2008. One (Chestnut Dale x GR226) was a Clapper B_3 family and one (Elk Knob x CB258) was a Clapper B_4 family. In June, 2012, the trees were inoculated with two strains of the blight (SG and Ep155) with two replications per strain and the height of each canker was measured the following November. Figure 1 shows the distribution, mean, and 95% and 99% confidence intervals for the mean canker height for each family at each location. There was a different distribution in terms of canker size even though all orchard sites were treated similarly. The arrows highlight the differences in mean canker height at two locations. Sara Fitzsimmons has had the same experience in PA with a single seed lot divided among three locations.

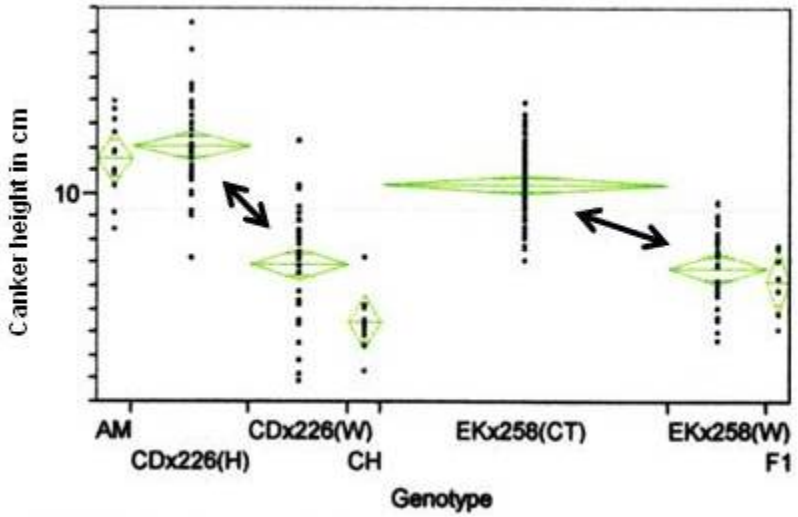


Figure 1. Mean and 95 and 99% confidence intervals of the mean for canker heights are represented by the green diamonds, with the midpoint of the diamond being the mean for each family. Each dot represents the mean canker height of an individual tree. AM, CH, and F1 were pure American, pure Chinese, and F1 trees included as controls in the orchards. CDx226 was a 'Clapper' B3 family and EKx226 was a 'Clapper' B4 family. (H) = Hurst Orchard (2200 ft elevation); (W) = Winterberry Orchard (4000 ft elevation) and (CT) = Cataloochee Ranch Orchard (5000 ft elevation) in Buncombe (H) and Haywood (W and CT) Counties, NC.

Differences in blight resistance between BC families. Three different families planted at the same site (Cataloochee Ranch) exhibited differences in blight resistance between the families (Figure 2).

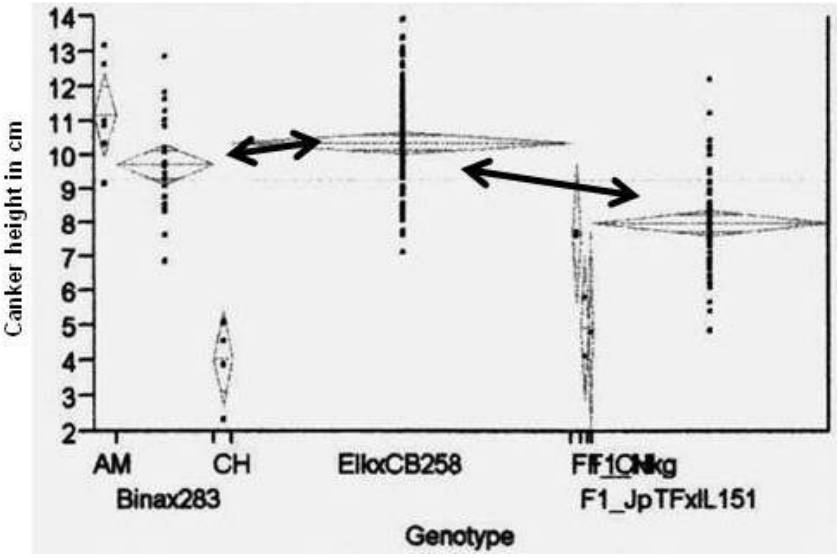


Figure 2. Canker heights measured at Cataloochee Ranch, Maggie Valley, NC in 2012. Mean and 95 and 99% confidence intervals of the mean for canker heights are represented by the green diamonds, with the midpoint of the diamond being the mean for each family. Each dot represents the mean canker height of an individual tree. AM, CH, and F1 were pure American, pure Chinese, and F1 trees included as controls in the orchards. The TFxIL151 family had a smaller canker size, on average, than the other two families (Binax283 and ElkxCB258). The American parent of the TFxIL151 family was the Ted Farmer tree in Avery County, NC. The source of resistance in this family is 'Clapper'.

Sisco speculated that the Ted Farmer American chestnut tree contributed to the blight resistance of this family. Crosses of Ted Farmer American by a Japanese chestnut tree also produced exceptionally resistant F_1 offspring (F1_Jp in Fig. 2). Sisco asked, "Do certain American chestnut trees contribute to blight resistance, particularly in 'Clapper' lineages?"

More evidence for the contribution of particular American chestnut trees to resistance can be found in R.B. Clapper's description of the origin of the 'Clapper' B_1 tree, as related in his article in the *Journal of Forestry* in 1963 (reprinted *TACF Journal* Spring, 2007.)

> One fortunate combination of parents used in 1932 was a Chinese chestnut, M16, P. I. 34517, introduced from Tientsin, China, in 1912, and an American chestnut sprout growing in the woods near the test orchard. The first generation trees were uniformly upright and rapid-growing. All were attacked by the blight; some of them died, no doubt from a combination of blight, poor soil, and close planting. However, three or four trees survived these unfavorable conditions until the test plot was abandoned in 1955.
>
> In 1935, we used the same Chinese parent, M16, in combination with another American chestnut that consisted of multiple sprouts from an old stump growing on a private lawn. This parent, **FP. 555**, grew in the open and was about 300 feet from one of our chestnut test plots. **The first-generation hybrids from this cross were uniformly more blight-resistant, and showed better tree form and a higher growth rate than the previous hybrids** (P. Sisco emphasis).

Clapper backcrossed the F_1 to the same FP555 American chestnut tree because he was so impressed with the particular combination of American and Chinese chestnut parents. It is

important to keep in mind that there are sometimes specific combinations of plants that produce superior offspring. In plant breeding this is called "specific combining ability".

Hebard and Sisco (Meadowview Notes, TACF Journal Spring, 1999, p. 15) reported on another exceptionally resistant 'Clapper' backcross family labeled "CC1xC", whose mean canker size was significantly smaller than the mean canker size of any other family measured that year. Preliminary genetic mapping of genes for blight resistance showed a locus from American chestnut contributing to resistance in the CC1xC family (T.L. Kubisiak and F.V. Hebard, unpublished data).

The CC1 American chestnut tree was already dead by 1999, but the Ted Farmer American chestnut tree is still alive and can be used for additional tests of the hypothesis that certain American chestnut parents can contribute to blight resistance in 'Clapper' backcross families.

Carolinas chapter seed orchard. The first plantings of B_3F_2 seed in a Carolinas 'Clapper' seed orchard were planted in 2012 to create **Restoration Chestnuts** adapted to the Carolinas. Each tree in the seed orchard has an American parent from North Carolina. Each block of trees is from a different Meadowview "line" and will be inoculated with blight strain SG in its 3rd year of growth. Each line in the Carolinas seed orchard is being designated by its corresponding Duncan Farm plot number at Meadowview. The lines that Fred Hebard has created all have the 'Clapper' B_1 tree as their source of resistance, but each line has been crossed by different American parents for 2 or more generations to avoid inbreeding when the F_2 crosses between lines are made. At Meadowview, the Duncan Farm plots contain the 'Clapper' lines while the Wagner Farm plots contain the 'Graves' lines. In the two Duncan and Wagner seed orchards each plot is replicated nine times.

Cytoplasm variation in American chestnut in the South. 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 F_1 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 (reviewed in Chase, C.D., Ribarits, A. and E. Herbele-Bors. 2010. Male sterility. p. 437-457. In: E.C. Pua and M.R. Davey (eds.), Plant Developmental Biology – Biotechnological Perspectives. Vol. 1. Springer-Verlag, Berlin.)

However, exceptional male-fertile F_1 progeny of American (female) x Asian chestnut (male) have been found in the southern U.S. Sisco et al. (Acta Hort, in press) have shown that this is the result of unusual cytoplasms 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 cytoplasms that result in male-fertile F_1s 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 chinkapin. Dane and Sisco have submitted an article to the TACF Journal describing the cytoplasmic diversity in American chestnut in South. It should appear in the Jan-Feb 2014 issue.

Sisco is using male sterile F_1 trees in the Carolinas seed orchard as checks. His reasoning is as follows:

1. TACF's goal is for **Restoration Chestnut** trees to be at least as good as F_1's (see the latest Meadowview notes in the TACF Journal).
2. Male-sterile F_1s will not contaminate **Restoration Chestnuts** produced in the seed orchard.
3. New sources of resistance can be introduced by collecting seed of F_1 trees x B_3F_2 trees selected for blight resistance. The offspring of this type of cross should have a high average level of blight resistance.
4. Phytophthora resistance can be added by screening these F_1 x B_3F_2 seed in tub tests, as described by **Jeffers, S.N.; James, J.B.; Sisco, P.H. 2009.** Screening for resistance to *Phytophthora cinnamomi* in hybrid seedlings of American chestnut. In: Goheen, E.M.; Frankel, S.J., tech. coords. Proceedings of the fourth meeting of the International Union of Forest Research Organizations (IUFRO) working party S07.02.09: Phytophthoras in Forests & Natural Ecosystems. Gen. Tech. Rep. PSW-GTR-221. Albany, CA: U.S. Department of Agriculture, Forest Service, Pacific Southwest Research Station: 188–194.
5. The F_1s should have one copy of every resistance gene – a good insurance policy in case resistance genes have been lost during the backcrossing process.
6. A new BC program can be initiated by using a different B_3F_2 selection at every backcross --- most offspring should have blight resistance. Thus, the numbers of trees to be grown out for screening can be small.

Genetic mapping of genes for resistance to blight and root rot. Sisco has been working with Teytyana Zhebentyayeva (Clemson Univeristy) to try and get a handle on the genetics of the resistance of Asian chestnut trees to Ink Disease incited by *Phytophthora cinnamomi*. Fred Hebard has been able to create a single family of about 1000 B_1 seed having 'Mahogany' chestnut as their source of resistance. Hebard has been able to do this because he has large F_1 trees that are 20 years old. In the Carolinas, Sisco is extending this analysis to families having 'Nanking' Chinese and Japanese chestnut as their source of resistance to see if resistance to Ink Disease is controlled by the same locus or loci as is operative in 'Mahogany' Chinese. There are some very nice Japanese specimen trees at Fort Defiance and Morrow Mountain State Park in western NC, planted around 1900 by people with a botanical interest.

Laura Georgi, The American Chestnut Foundation, Meadowview

DNA markers at Meadowview. She is interested in developing assays that incorporate molecular markers in a cost effective manner that are applicable to a breeding program. The potential applications for molecular markers include:

- Identification of cultivars, species and hybrids
- Parentage analysis
- Discrimination of homozygotes from heterozygotes
- Marker-assisted selection
- Evaluation of genetic diversity
- Intellectual Property Protection

Last year she talked about SNPs; this year she has looked at SSRs. She and Hebard have gone through the material generated by the NSF Fagaceae project of the genotypes of mapping

population parents. They identified a number of SSRs that had large allelic differences that were potentially discriminated among mapping parents. She selected a number of SSRs for evaluation. She used SSR maker CmSI0009 to look at vegetatively propagated grafted material from a number of different Chinese sources ('Vanuxem', 'Nanking' and 'Meiling'). She noted putative differences among the seven 'Nanking'. She also began looking at some SSRs in the blight QTLs.

Graves B_3s– major seed orchard parents. Many lack the 'Mahogany' alleles at these two markers linked to QTL on B and G.

- Recombination between locus and marker
- If marker is within 10 cM, linkage should be maintained in >70% of B_3s, assuming perfect selection
- If UNlinked, expect frequency of 1 in 8 in the B_3
- 10/19 for CmSI0509; 53%
- 8/19 for CmSI0614; 42%
- Caveat: Fragments that appear to be the same size (at this or any resolution) may not be the same
- Multilocus trait (less than perfect selection)
- Position(s) of the QTL may not be properly determined

Georgi has a lot of SNP data and she could not resist that opportunity to play with it. There was a lot of transcript data that came out of the NSF program from American and Chinese chestnut. She has been asked if she has any markers that can discriminate between American and Chinese trees. The answer was 'no' and that led to the following study using Galaxy, a Penn State tool for analyzing next generation sequence data.

Galaxy workflow

- Upload contig assemblies from www.fagaceae.org
- Upload sequence reads from the European Nucleotide Archive
- Groom and trim read sequences
- Map CC reads to AC contigs and AC reads to CC contigs (Bowtie or BWA)
- Filter and convert SAM output to BAM
- Merge BAM files and Pileup
- Filter Pileup

Started with Bowtie (although it does not do gap alignment) figuring she wasn't interested in indels because these are 454 reads with artifactual indels, but realized belatedly that BWA would do a much better job of aligning for that very reason. She will have to go back and see what extra information she can get out of the BWA results, but she continued with the Bowtie output. She eventually found 43 contigs with reciprocal best match; these typically have more than one SNP.

For 42 contigs, where there is a homologous pair of American and Chinese, she is using the Peach genome to identify possible/probable locations of introns. These are expressed sequences so she does not know the genomic organization but she is pretty sure there are introns. Designing markers from those will have much higher success rate if she knows where the introns are.

Downstream analysis

- Align to related genome to locate probable introns
- Design primers and evaluate
    - Tetra-primer ARMS?
- Synonymous/Nonsynonymous
    - Conserved/Nonconserved/Nonsense

Cryphonectria transcripts in canker transcriptome

- Galaxy
    - Upload Crypa v2 genome assembly from JGI (the ribosomal repeat region on in the *C. parasitica* genome isn't annotated as such on the JGI DOE browser)
    - Map reads with Tophat (gapped alignment)
    - Evaluate with Cufflinks
        - Gene annotations would REALLY help
        - Reformatted JGI .gff to .gtf: hasn't worked yet
- Huge number of transcript reads map to scaffold_6:559,000-578,000
- "SG2" read depth over 1000X
- Looks like a tandem repeat—it is ribosomal
- Looks like a COLLAPSED tandem repeat
    - Based on SG2 read coverage, approximately 30 ribosomal gene copies

Fred Hebard, The American Chestnut Foundation, Meadowview

Breeding for Phytophthora resistance. From the perspective of TACF, the big question is 'How is *Phytophthora cinnamomi* resistance incorporated into the breeding lines?' Progress reports of blight resistance based on measurements of orchard progeny of the BC_3F_3 can be found in the TACF Journal (Meadowview notes) for 2011 and 2012. In the first year of testing 17% of the individuals qualified as highly resistant. In the second year, that number dropped to 4%. In the third year of testing (2013), there should be an increase because the weather has been cool and wet.

Hebard is certain that we should get resistance to the level of F_1s which may be good enough for reproduction in the forest. Right now, the BC_3F_2 orchards are still being culled. Assuming we can fix blight resistance in the BC_2F_2, that won't be enough for some in the southern U.S., as there is no *P. cinnamomi* resistance. *P. cinnamomi* largely eliminated American chestnut in the coastal Piedmont in the 1800s. Paul Sisco heads up the southern breeding program and Sisco proposed breeding for *P. cinnamomi* resistance. Joe James, a retired surgeon living in Seneca, SC noticed *P. cinnamomi* killing many of his trees. James and Steve Jeffers at Clemson University developed a method for screening chestnut seedlings for *P. cinnamomi* resistance. Testing to date, coupled with the genetic analysis of Tetyana's work, indicates 'Graves' line of blight resistance has retained Phytophthora root rot resistance in about 25% of the third backcross trees. Extrapolating these data into the Hardy-Wienberg equation for the F_2 population, Hebard expects a frequency of about 25% of the trees will retain resistance to Phytophthora root rot. These trees will be heterozygous for *P. cinnamomi* and it appears to be co-dominantly inherited.

'How do we incorporate Phytophthora resistance (PRR) into TACF's breeding lines?' PRR appears to be a single gene. Using classical methods, PRR resistance can be fixed in two more

generations and moved into all the genetic backgrounds so that we can retain all the diversity. First generation—move into all the backgrounds and the second generation, make it homozygous. This will be done with open pollination because we are dealing with such a large number of trees. The question is how many trees do we need at the BC_3F_3 and BC_3F_4 level? Our populations are going through a bottleneck during the breeding process and the lowest point of the population size of the bottleneck size is at the F_1 –be it the BC_3F_1 or BC_4F_1. State chapter breeding programs are adding new American backgrounds to the breeding stock. Additionally, advancing to new backcross generations reduces the inbreeding coefficient between lines. These goals are met largely through the state chapter system. The Meadowview material does not have enough genetic diversity to persist for a long time. A long while ago, Hebard estimated that they would have to increase their population size by a factor of nine between F_1 and F_2 and he did a lot of simulations of inbreeding through F_4 to answer questions as to how to arrange trees in breeding orchards. It ended up that the harmonic mean of a population size between F_1 and F_4 is adequate to give a fairly good estimate of the effective population size, another metric of genetic diversity. Going from F_2 to F_3, you only have to increase the family size by a factor of four (in addition to the factor of nine increase from F_1 to F_2), holding all sample sizes constant (an important component of the breeding process). This is a factor of 36 increase over the F_1 population size which we are treating as 20 (American lines). That is only 720 trees at F_3 and F_4 that we need to select in order to capture most of the genetic diversity. This is achievable with reasonable population sizes.

Karyn Willyerd, New Mexico State University

Investigation of LysM in *C. parasitica*—introductions and knockouts. The Lysin Motif (LysM):

- Ubiquitous protein motif
- Was first identified in bacteria
    - PG hydrolase- cell wall degradation
    - Approximately 40 amino acids
    - Identified by YG consensus (YXXXXGXXHy) within first 16 residues
- Pathogenic mechanisms
    - PG recognition- bacteria
    - Chitin recognition- eukaryotes

Chitin is a β-1,4 linked n-acetyl glucosamine. It is known as a PAMP—pathogen associated molecular pattern and it is recognized by host PRRs (pattern recognition receptors) which initiate pathways for host defense response. Chitin is a major component of fungal cell walls.

Fungal LysM effectors:

- Secreted effector proteins shown to establish virulence
    - *Cladosporium fulvum* Ecp6 (de Jonge et al. 2010)—tomato pathogen
    - *Magnaporthe oryzae* Slp1 (Mentlak et al. 2011)—rice pathogen
- Compete for binding of chitin
- Initial plant defenses are overcome and fungal invasion occurs

They have isolated five putative LysM proteins:

- LM12--Secreted effector protein, chitin recognition
- LM83--Secreted effector protein, chitin recognition
- LM25--Protein-protein interactions, vegetative incompatibility
- LM47--Ergosterol regulation (Arv1), protein-protein interactions
- LM69--Chitin recognition, mycelial development

This protein acts to shield the chitin from the host pattern recognition receptors so the plant does not initiate plant defense responses.

Willyerd is most interested in LM12 as it shares 30% homology to Ecp6 and Slp1 and it may act as an effector protein. While they do not predict that all the proteins are involved directly in pathogenicity, all will be tested to see if they have any other function.

One way to characterize the proteins is through gene knockouts:

- PCR based method—amplify upstream and downstream linking regions and join to half the hygromycin cassette
- Transformation into fungal spheroplasts
- Replace gene of interest with hygromycin cassette
- Confirm knockouts by Southern Blot—these knockouts will be done in both Ep 155 and the ΔCpKU80 strain

ΔLM12 ΔLM25 give a phenotype very similar to wild type, while ΔLM69 gives a phenotype with slightly reduced pigmentation and sporulation. ΔLM47 produces a very severe phenotype and when inoculated into dormant chestnut stems, it is avirulent.

Future work:

- Continue screening transformants
- Confirmation of ΔCpKU80/ΔLM knockouts
- Virulence assays
- Complementation
- Functional characterization

Megan McClean, New Mexico State University

Functional characterization of LysM. There are 5 proteins putatively containing LysM domain in *C. parasitica*

- LysM is important in virulence of other fungal plant pathogens
- Knockouts to compare virulence
- Functional characterization of LysM containing proteins
- Do these proteins act as effector proteins?

Plant defenses to fungal pathogens:

- Plants use pattern-recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs) to activate the initial immune response.
- The PRR of rice (chitin elicitor binding protein, CEBiP) recognizes chitin oligosaccharides as a PAMP of *M. oryzae*. This activates signaling for the initial immune defense to the pathogen.

LysM-containing proteins:

- LysM containing proteins in other fungal plant pathogens are effector molecules
- These effectors act as a competitor with PRRs by binding to chitin oligosaccharide

McClean is attempting to determine functions of these protein in *C. parasitica*—are they secreted and do they bind chitin? In order to do that she is in the process of "tagging" the proteins. This involves using a flag tag (only 8 aa long) on the designed primers for all five of the genes. The sequence for the flag tag is attached to the reverse primer. She then conducts PCR using Ep 155 genomic DNA, followed by sequencing. Once they have a confirmed product that has the tag, it is cloned into an expression vector and continue forward with fungal transformations that will be confirmed with Western blots. After a flagged protein is obtained, she will conduct assays for secretion and chitin binding. Before she gets started with the secretion assays, she has conducted research on each of the genes that will give some predictions. LM12 and LM83 both have evidence for signal proteins. For secretion assays:

- Liquid cultures of FLAG-tagged transformants will be used.
- Mycelium will be separated from the media using filtration.
- Precipitate protein from filtrate and use SDS-PAGE and Western Blot to detect tagged proteins.

For the chitin binding assay:

- Precipitate protein with chitin along with other polysaccharides (to select for specificity to chitin)
- Spin down, separate pellet and supernatant. If the proteins are present in the pellet she can show that the proteins were able to bind with the polysaccharide.
- Check for presence of protein using SDS-PAGE
- Protein found in pellet-binds to polysaccharide
- Protein found in supernatant-does not bind

Current progress:

- LM25 gDNA-western blot to confirm FLAG-tag presence
- LM12 gDNA, LM69 gDNA, LM83 gDNA and LM25 cDNA-fungal transformation stage
- LM47 gDNA, LM12 cDNA, LM69 cDNA, and LM83 cDNA- PCR stage, troubleshooting for cDNA

Didi Ren, New Mexico State University

Transcriptional changes in the absence of VIB-1, a component of the incompatibility-mediated cell death pathway.

vib-1:

- Mediator of heterokaryon incompatibility
- Regulator of conidiation
- Reduces hyphal growth
- Required for programmed cell death response in *Neurospora crassa.*

Ren wants to see if *vib-1* in *C. parasitica* functions similarly to *N. crassa*. The *⊿Cpvib-1* strain produced increased pigmentation, profuse sporulation and reduced hyphal growth.

In a vc test, Ep 155 and EU 1 are incompatible. However, *⊿Cpvib-1* and EU 1 are compatible. In a virulence assay on dormant chestnut pieces, *⊿Cpvib-1* barely grew compared

to Ep 155. The *⊿Cpvib-1* strain grew even less than Ep 713. Thus, *vib-1* plays an important role in the virulence of *C. parasitica*.

Hypothesis: The role of CpVIB-1 as a mediator of incompatibility related cell death requires direct and indirect interactions with other proteins that may include the products of the *vic* loci themselves. To prove this hypothesis, she did a transcriptome comparison between wild type strains and its isogenic *⊿Cpvib-1* strain. Renn used an Illumina Truseq RNA kit to prepare her library. The reads were aligned to the transcriptome in JGI suing bowtie2. The annotations were conducted with Blast2GO. The database results were:

- Over 200 million reads
  - 60% aligned to the reference transcriptome
  - 90% hit the genome
  - 40% in KEGG (database of possible metabolites)
- Total of 11,609 predicted genes
- 697 were significantly down regulated
- 181 were significantly up regulated

Most of the down and up regulated genes were involved with lipids, amino acids and carbohydrates.

Concerning authophagy and cell death:

- ATG-1 (one of 24)—a cvt (cytoplasma-to-vacuole targeting) was significantly up regulated.
- Bap-31-like protein—apoptosis and caspase-like activity was significantly down regulated.

Secreted proteins play an important role in the virulence of *C. parasitica*. About 11% of the genes encode a signal sequence for secretion and 167 of them are altered significantly. Seven belong to aspartic proteases. She wants to know if there are other transcription factors similar to vib-1 that can act directly or indirectly to regulate transcription. Eighteen (7 up and 11 down regulated) of 401 are unidentified proteins that may be involved with transcription. Pro-1 is required to sporulation and it is up regulated.

Conclusions:

- Lack of Cpvib-1 leads to significant changes in the expression of many genes, notably those encoding transcription factors and secreted aspartic proteases.
- This study provides the foundation for exploring the role of *Cpvib-1* in mediating the incompatibility and changing phenotype.

Jeanne Romero-Severson, University of Notre Dame

Identity-verified chestnuts: progress on the chestnut pedigree project. She is developing genetic testing to be able to determine species of chestnut, and if a tree is a hybrid, how long ago did it become a hybrid.

The role of genetic testing in chestnut improvement and restoration:

- Verify cultivar identify
  - Valid performance evaluation
  - Predictable characteristics
  - Legal protections

- Detect recent ancestry
    - Identify interspecific hybrids
    - Predict genetic value in descendants
    - Predict the best parents for crossing

Ancestry project design

- Nuclear and chloroplast genomes
- Distinguish three levels of variation
    - Among individuals within "ethnic groups" (Individual specific)
    - Among ethnic groups within species (ethnic specific)
    - Among species (Species specific)

Project design (shown below)—she is developing genetic markers to detect both the chloroplast and the nuclear genome. These two pieces of information together are very informative. This needs to be done to an almost a forensic standard, because we are dealing with five different species. For an outcrossing forest tree, every tree is different. We need to be able to distinguish one chestnut population from another. We need to distinguish the genetic variance that occurs within a species as well as between species. This is why one or two markers are not enough. If we are dealing with orchards, why is one orchard doing better than another. We need a database. Crossing of species over thousands of years make cultivars highly related.

Collect 50 unrelated individuals from each chestnut species	
Chloroplast Genome	**Nuclear Genome**
Identify polymorphic regions by sequencing	Screen existing mapped EST-SSR markers for nuclear polymorphisms
Screen for species specificity	Design primers
Select those regions that distinguish the five species*	Test for amplifiability, reliability and polymorphisms
Determine optimum number of markers	Determine optimum number of markers
Determine Ancestry	

**C. sativa, C. mollissima, C. crenata, C. dentata* and *C. pumila* (including *C. pumila* var. *ozarkensis*)

The chloroplast genome is being sequenced. Romero-Severson has sequenced the entire single copy region for *Quercus rubra* and based on that, a lot of chloroplast properties can be used for any tree in the Fagaceae. She is sequencing polymorphic regions to determine species-specificity in the chloroplast. This can be done because the chloroplast is haploid lineage; it does not recombine. This information is coupled with nuclear genomic information from EST-SSRs. These make very good heterologous markers. They will work across all species.

Total collection to date

N	English Name
60	American (many related)
46	Chinese (some related)
34	European (some related)
17	Japanese (some related)
36	Ozark chinkapin

0	Allegheny chinkapin
2	*C. henryii*
2	*C. seguinii*
33	Hybrids, unknown, unidentified

Romero-Severson's screening panel is: 6 European; 2 Ozark chinkapin; 3 American; 6 Japanese and 7 Chinese.

Possible species-specific sequence differences in an intergenic regions—the HK region may provide species-specific diagnostics for *C. mollissima*, *C. crenata* and *C. pumila* var. *ozarkensis*. The idea is to do a minimal amount of screening for a maximum amount of identity determination.

Ancestry project design:

- Chloroplast genome
    - Sequence additional intergenic spacer regions polymorphic in the Fagaceae to distinguish *C. sativa, C. dentata, C. mollissima, C. crenata, C. pumila*
    - Straightforward species-specific chloroplast test
- Nuclear genome
    - Genotype with a set of 20-30 EST-SSR
    - Mapped in Chinese chestnut
    - Informative across all five species
        - Amplify one and only one amplicon in all five species
        - High PIC in all five species

EST-SSRs

- In or near expressed genes
- Contain SSRs
- Excellent for cultivar identification
- Work across many species

1. Capture mRNA for expressed genes and make cDNA
2. Sequence cDNAs Expressed sequence tags (ESTs)
3. Assemble contigs
4. Screen for EST-SSRs
5. Screen existing mapped EST-SSR markers for nuclear polymorphisms
6. Design primers
7. Test of amplifiability, reliability and polymorphisms
8. Determine optimum number of makers

Annotation for contigs containing the 11 EST-SSR markers used in a previous study

CONTIG	MARKER	ANNOTATION
CC454_CONTIG350_V2	Cm350	Syntaxin-like protein
CC454_CONTIG353_V2	Cm353	WRKY Transcription factor, putative
CC454_CONTIG2033_V2	Cm2033	Homeobox protein knotted 1-like
CC454_CONTIG3815_V2	Cm3815	Nucleobase-ascorbate transporter 3-like
CC454_CONTIG8679_V2	Cm8679	Ubiquinone biosynthesis protein coq-8, putative
CC454_CONTIG9659_V2	Cm9659	No hits found

CC454_CONTIG13321_V2	Cm13321	Metal tolerance protein 9-like
CC454_CONTIG13496_V2	Cm13496	Pleckstrin homology domain-containing protein 1-like
CC454_CONTIG12493_V2	Cm124933	Hypothetical protein
CC454_CONTIG27500_V2	Cm27500	Auxin response factor 2-like
CC454_CONTIG34644_V2	Cm34644	Conserved hypothetical protein

Romero-Severson will query all the EST-SSRs, equally distributed among the 12 chromosomes, until she has a good enough set where she can be 95% confident of a species, within the information in their database. She requested additional geo-referenced samples. She would like 20 dormant twigs. Email her and she will send instructions for sending material.

John Carlson, Pennsylvania State University (submitted report)

Update on The Chestnut Genome Project. This project was supported by The Forest Health Initiative (http://foresthealthinitiative.org/) to evaluate the role of biotechnology in addressing forest health challenges, starting with American chestnut restoration. The primary goal for the Chestnut genome project is to produce a high-quality reference sequence for the genome of *Castanea mollissima* cv 'Vanuxem'. The Chestnut genome sequencing project builds upon the results of a previous NSF-sponsored project "Genomic Tool Development for the Fagaceae" (www.Fagaceae.org) and upon breeding materials developed by the American Chestnut Foundation. Direct participants in the project include John Carlson, Stephan Schuster, Charles Addo-Quaye, Lynn Tomsho, Daniela Drautz, Lindsay Kasson, Tyler Wagner, and Nicole Zembower at Penn State University, with Meg Staton, Abdelali Barakat, and Bert Abbott at Clemson University.

Progress with genome project. Stage 1, first round of sequencing: In 2010 and 2011, the project produced and assembled great depth of "shotgun" (random DNA fragment) genomic DNA sequence resulting in:

- 55.3 Gbases of gDNA sequence from 454 and Illumina next generation platforms
- Sanger sequences of the ends of BAC clones covering the entire chestnut physical map
- Assembly of 925 Mbp on 1,147,939 contigs and 587 Mbp on 51,766 scaffolds
- Average scaffold length of 52Kb.
- Aligned over 96% of 48K Chinese chestnut transcript contigs from Fagaceae project.

Stage 2, second round of sequencing: The large number of contigs and scaffolds produced in the first assembly suggested either high levels of heterozygosity or contamination in our original genomic DNA libraries. In 2012-13 we engaged in new sequencing and assembly resulting in:

- Preparation of new genomic DNA library from a single *Castanea mollissima* cv Vanuxem ramet in the TACF breeding orchard
- Produced 60 Gb genomic DNA sequence by Next Gen sequencing technologies
- 724.4 Mp of sequence assembled in 41,270 scaffolds with median length 39.6 Kb
- Complete assembly size predicted by = 1,844.3 Mb / 1C genome.
- 38,268 genes predicted by 'MAKER' algorithm, with average of 4.6 exons/gene.

Stage 3, sequencing of blight resistance QTL: We have sequenced pools of BAC clones which cover the three blight resistance QTL, selected from the Chinese chestnut physical map. The QTL sequences were assembled by Meg Staton at CUGI and analyzed by the A. Abbott team:

- We obtained 518.6 Mp of sequence using 454 FLX+ and 4.7 Gb by Miseq technologies.
- 95% of the sequence reads were used in assembly of QTLs into contigs and scaffolds.
- **QTL *cbr*1** on Linkage Group (LG) was B assembled into 214 scaffolds containing 6.8 Mb of DNA sequence in which 432 genes were identified and annotated.
- **QTL *cbr*2** on LG F assembled into 128 scaffolds containing 4.1 Mb of DNA sequence in which 219 genes were identified and annotated.
- **QTL *cbr*3** on LG G assembled into 53 scaffolds containing 3.0 Mb of DNA sequence in which 131 genes were identified and annotated.
- Fifteen genes were annotated with the GO function term "defense response" and were thus selected as high priority candidate genes for blight resistance.
- The QTL defense response genes included the annotations: transcription factor, CC-NBS-LRR resistance protein, RNA-binding protein, 14-3-3-like protein, endopeptidase, protease, disease resistance, glycosyltransferase, receptor-like kinase, and histone-methyltransferase.
- Manuscript in preparation.

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

***Cryphonectria parasitica* vegetative incompatibility genes.** When he first came to this meeting, he was intrigued by non-self recognition in the fungus and how *C. parasitica* is able to recognize itself. Barriers or non-fusion of hyphae are very important in virus transmission. No barriers allows for virus transmission while incompatibility or barriers does not allow for virus transmission. What are the genes that are controlling this process? A number of things have come together over the past few years that have helped establish the elements enabling the identification of *vic* genes. Those are:

- Identification of 64 *vic* genotyped tester strains by Michael Milgroom and colleagues
- Genetic linkage map developed by Milgroom and Tom Kubisiak
- Genome sequence by the Department of Energy
- Predicted polymorphism at *vic* loci based on work with *Neurospora* and *Podospora*

As a background, Ep 155 and Ep 146 (brown-pigmented) differ at loci 2, 4, 6 and 7.

vic loci 1234-67
EP155 2211-22
EP146 2112-11

*Vic*2 was identified because it was mapped on the linkage map. Taking some of the sequenced linkage markers, the scaffold was identified from the genome assembly. The 454-reads were mapped to the reference scaffold and gaps (that would indicated polymorphisms) were identified. There were two polymorphic open-reading frames for vic-2 (patatin-like protein and sec9). These two open reading frames are always linked.

*Vic*4 is quite interesting as it does not prevent transmission of virus. If there are different alleles at the *vic*4 locus, there is a slow formation of barrage but it does not prevent virus transmission. *Vic*4 is located on scaffold 4 and in this case it is not polymorphic but idiomorphic—completely different genes. For allele 2, it is a kinase gene and for allele 1 it is an entirely different that has the hallmarks of heterokayon incompatibility genes in *Podospora*.

*Vic*7 is on scaffold 6. It contains a het-domain gene that is found in most heterokaryon incompatibility genes in *Neurospora* and *Podospora*.

Vic6 has two open reading frames on scaffold 3 (*vic*6 and *pix*6, a partner of *vic*6). There is quite a bit of polymorphism with the het-domain for vic6. The entire N-terminal region of pix6 is conserved and all the polymorphism is in the C-terminal region.

They were able to disrupt *vic*2-2, *vic*6-2 and *vic*7-2, and they looked at barrage formation that was affected by the deletion of these alleles. Disruption of these alleles led to:

- No phenotypic changes
- No loss of barrage formation
- Increase in virus transmission for the *vic*2-2 and *vic*6-2 mutants when serving as recipients and for the *vic*7-2 when serving as the donor

This raised the question of whether disruption of both alleles would abolish barrage formation. This led to a detailed characterization of the vic6 locus.

With regard to the interaction between vic6 and pix6, the disruption of both alleles for vic6 (either *vic*6 or *pix*6) eliminated programmed cell death and any resistance to virus transmission. The incompatible reaction for vic6 is controlled by non-allelic interaction between *vic*6 and *pix*6.

Vic genotyping for *C. parasitica* strain Ep155 and resequenced strains:

***vic* loci 1234-67**

EP155 2211-22
EP146 2112-11
EU55 1221-22
EU31 1211-22
EU60 2221-22
EU40 1122-11

There was very little information on *vic*1 and *vic*3, so EU55, which differs from Ep155 was both vic1 and *vic*3, was resequenced. Also, EU31 and EU60 which differ at *vic*1 and *vic*3, respectively, also were resequenced. They also looked at EU40 which differs at all 6 loci.

*Vic*1 is on scaffold 5 and they were able to identify a marker, E19.

Summary for putative vic1 locus:

Inspection of the EU-31 reads mapped to EP155 scaffold 5 in a region that contained *vic1* linkage markers resulted in the identification of polymorphism located just 3.19 kb from the linkage marker E19 0825. The polymorphic region consists of a gene encoding a 771 amino acid HET domain-containing protein and an adjacent gene that encodes a 172 amino acid long hypothetical protein that contains a DUF (conserved domain of unknown function) domain. These genes were designated *vic1a*-allele 2 (*vic1a-2*) and *vic1b*-allele 2 (*vic1b-2*).

The *vic1* locus candidate alleles *vic1a*-2 and *vic1b-2* were disrupted in the DK80 non-homologous end-joining mutant strain of EP155. While strains DK80 and EU-31 undergo an incompatible reaction, forming a clear barrage when paired, the Δ*vic1a-2* disruption mutant

was compatible with EU-31. In addition, deletion of *vic1a-2* resulted in an increase in virus transmission from 0-10% to 100% when the mutant served as the virus recipient and EU-31 served as the virus donor and from 5/20 to 20/20 when the mutant serves as the donor. These combined results provide strong evidence that the *vic1a-2* gene is a component of the *vic1*-locus.

Disruption of the *vic1b-2* allele resulted in a reduced growth and altered morphology phenotype. This altered phenotype made scoring of barrage formation and virus transmission difficult. However, the growth and morphology alterations were ameliorated when the mutants were grown on an enriched medium. Under these conditions, the Δ*vic1b-2* mutant strain showed reduced barrage formation and increased virus transmission from 20-30% to 100% when serving as the recipient. Studies are in progress to determine the full contributions of *vic*1a and *vic*1b allelic and possible non-allelic interactions to the incompatible reaction and restriction to virus transmission.

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.

Pam Kazmierczak, UC-Davis

***Cryphonectria* hypovirus 1 protease p29 is a determinant of virus-induced vesicle accumulation within host cells.** This work is on the prototypic virus, CHV1, and the majority of the p29 work was conducted by Debora Jacob-Wilk. There are commonalities among three genes down-regulated by the virus: cryparin (type 2 cell surface hydrophobin), laccase (polyphenol oxidase) and MAT-1 pheromone. They all contain the signal sequence for secretion and Kex2 processing, an endoprotease that resides in the trans-Golgi network and finishes the protein prior to secretion outside the cell. The replication complex of CHV1 was found to be of trans-Golgi origin of *C. parasitica*. There is anecdotal evidence that CHV1 disrupts the secretion of cryparin. The isolates used the study were:

Ep 67	MAT1, uninfected
Ep 155	MAT2, uninfected
Ep 802	MAT1, CHV1-infected
UEP1	MAT1, CHV1-infected
CHV2-155	MAT2-, CHV2-infected (does not have a coding domain for p29)

Kazmierczak noticed that hv isolate, Ep802 accumulates more cryparin than virulent isolate, Ep67. She wanted some quantitative data on cell wall accumulation of cryparin, looking at both cell wall cryparin and intercellular cryparin. Subcellular fractionation, cryparin-green fluorescent protein (GFP) fusion and Western blot studies confirmed that vesicles containing cryparin copurify with the same fractions previously shown to contain elements of the viral replication complex and the trans-golgi network resident endoprotease Kex2. This vesicle fraction accumulated to a much greater concentration in the CHV1-infected strains while in Ep 67, cryparin is rapidly secreted where it re-associates with the cell wall. How does the virus cause this disruption in secretion and why choose p29? Single-stranded viruses often use host secretory membranes for replication and they often use nonstructural proteins to enhance build up the replication complex. p29 is a good candidate because it is papain-like protein, similar to HC-Pro, a helper component protein in Potyviruses to which CHV1 is related, and it is a multifunctional protein (reduction in symptom expression, sporulation, pigmentation).

Constructs used to illustrate role of p29:

- All plasmids were transformed into Ep 67
    - pXH9 full-length L-dsRNA cDNA clone
    - pXHΔ1 (full infectious clone without p29)
    - p29 PCR fragment cloned into PCPXHY1 in both orientations.

There was no p29 expressed in Ep 67 nor in CHV1pΔ29 but it was expressed in Ep 802, and the PCPXHY1 construct (in the forward orientation but not the reverse orientation). They found that where the virus was present and where p29 is present, there is an accumulation of the vesicle fraction. She compared Ep 155, with and without an anastomosed CHV2 and found that vesicle accumulation was similar between Ep 155 and the CHV2-Ep 155.

Pulse-chain analysis showed that the rates and amount of cryparin being secreted by the CHV1-infected strains was much lower than in non-infected strains and the dwell time of cryparin within the cell after labeling was significantly greater in the CHV1-infected strains than in non-infected ones, suggesting that the virus perturbs a specific late trans-golgi network secretory pathway resulting in buildup of a key protein important for fungal development.

Conclusions:

- p29 is important for vesicle accumulation in virus-infected strains.
- Vesicle accumulation is important because that is where the virus replicates.
- CHV2 doesn't have p29 but it still accumulates some vesicles.
- All viruses need membranes on which to replicate and there is an unknown protein in CHV2 that is helping to build the replication factors; however, there is not as much vesicle accumulation in CHV2 and in CHV1.

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, M. Milgroom, Cornell University). 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 3318 cankers have been identified in the 12 plots. Four-hundred, forty cankers on living trees were sampled in May 2013; 135 were newly discovered.

General observations:

- When the 12 permanent plots were established in 2001, there were 517 living stems included in the study. In 2013, 51% of the original stems in the Disease Center plots

remained alive compared to only 18% and 13% for the Disease Front and Beyond the Disease Front plots.

- 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 77% in 2012. WS-2 and WS-3 were found at rates of 2% and 16%, respectively.

Evaluation of *Cryphonectria parasitica* from a chestnut plantation on Bunner's Ridge, Marion County, WV. M. Haggblade (Pomona College undergraduate student summer intern). Approximately 400 chestnut trees from various sources (Michigan, irradiated and a few other sources) were planted (1988-1991) in a cleared area near Morgantown, WV. In 1998, a French exchange student, Noèmie Biegbeder, undertook a study of the natural infections that arose on the trees. As part of her study, she isolated *C. parasitica* from the cankers, transmitted hypovirus to each of the isolates (using Dave Huber's Hv isolates) and treated the cankers from which she had obtained the isolates in an effort to control the infections. For the past 15 years, the site has remained undisturbed. Tree survival and cankers with unusual morphology prompted further study at the site.

In 2011, a student from Pomona College (Marlene Haggblade) categorized 50 cankers from the site based on canker morphology (25 as virulent and 25 as hypovirulent). *C. parasitica* isolates from all 50 cankers appeared to be virus-free. Failure to detect hypovirulent isolates prompted further sampling of cankers on larger trees with significant callus. In contrast to the first sampling, four of twelve cankers yielded hypovirulent isolates. The putative hypovirulent isolates that were tested produced a single band of dsRNA. Some of these isolates were inoculated into living stems at the WV site and at the Savage River State Forest, Grantsville, MD to assess growth and sporulation. One isolate was much less pathogenic than V controls.

We have recovered hypovirulent strains from cankers at the site but principally from the few trees that remain and had received treatment by Biegbeder in 1998. There is little evidence for hypovirus spread in the stand. Thus, we conclude that the general improvement in the condition of some stems in the stand cannot be explained by the earlier, limited hypovirus introductions.

Anita Davelos Baines, University of Wisconsin, La Crosse

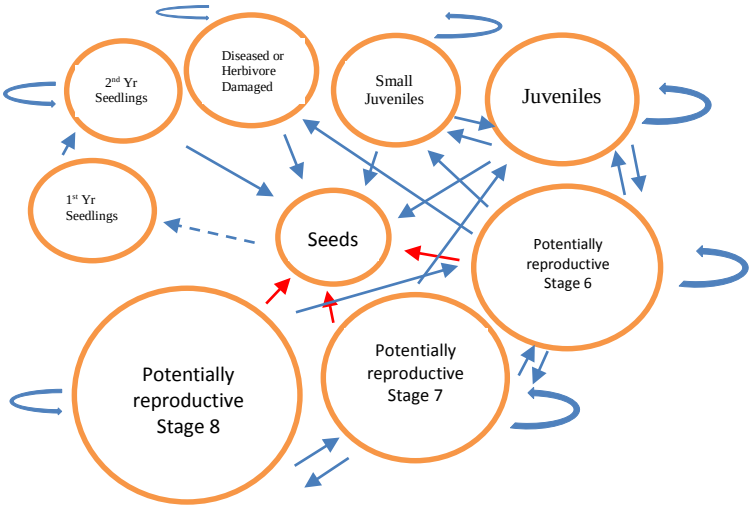
Modeling and analysis of a density-dependent structured population model for American chestnut. The motivation for this work is:

- Chestnut blight is a classic example of how introduced pathogens can alter host population biology and overall plant community dynamics.
- The 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 the chestnut tree and the fungus.
- If this hypovirus creates a callus that successfully halts canker 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 2004, she and Andrew Jarosz (Michigan State University), constructed a linear matrix model to study the dynamics of healthy, diseased and hypovirus-infected American chestnut populations. They found that the population growth rate, λ, was not statistically different than unity in all six (two healthy, two diseased and two hypovirus-infected) chestnut populations sampled in the northwest lower peninsula of Michigan. However, the long-term stage structure of hypovirus-infected populations was very similar to that of healthy populations, which were both different than that of diseased populations. This suggests that hypovirus-infected populations were truly recovering from a demographic perspective.

Model schematic—density dependent feedbacks:



Model development:

- That λ is not statistically different than unity in all six chestnut populations strongly suggests that the population is actually at some equilibrium, and this equilibrium is elicited by density-dependence in one or more stage in the life-cycle.
- It has been observed that seed and/or seedling establishment is density-dependent in many plant and tree populations, including American chestnut.
- Townley et al. (2012), Rebarber et al. (2012), Smith and Thieme (2013) and Eager et al. (2013) have studied mathematically how outside perturbations influence the size, demography and health of equilibrium populations for general population models with simple assumptions on density dependence.
- We will assume that stage transitions such as tree growth, shrinkage are density independent, as in Davelos and Jarosz (2004). Therefore, our model uses the parameter values in the three matrices in that paper.
- We assume, as in Davelos and Jarosz (2004), that seed production is density independent. Thus, we assume that the number of seeds produced per tree per stage

(as a proportion of the seeds produced per tree in other stages) is the same as in that paper.

- If seed establishment were assumed to be density independent, then our models completely reduce to those in Davelos and Jarosz (2004).
- For most plant and trees species, density-dependent feedbacks negatively influence seed and seedling establishment.
- In this model, we assume that seed establishment is density-dependent.
- For many plant and tree species, it is unreasonable for us to assume that seed establishment is suppressed by density-dependent feedbacks from newly created seeds alone, so we assume that both seeds and adult conspecifics can negatively influence seed establishment.
- However, in many cases, these density-dependent feedbacks elicit populations that are robust to external perturbations/disturbances.

Theorum: If the establishment probability of a seed in the complete absence of any other competing seeds is sufficiently large, then the population will persist, regardless of its initial size and demography. Furthermore, the population will converge to an equilibrium population that we can compute, which is robust to perturbations/disturbances.

Interpretation of results for American chestnut:

- The long-term persistence and size of the American chestnut population depends on the stability radius p_e of the population and its relation to the seed establishment probability g0 in the absence of other competing seeds.
- Specifically, we need $p_e < g_0$ for persistence. Thus, if p_e increases the population is more likely to go extinct in the long run.
- Thus, if we can determine how p_e changes with respect to changes in disease incidence we can determine the impact of disease on the population size and persistence of the American chestnut population.
- For the matrices in Davelos and Jarosz (2004), p_e is an increasing function of both the amount of fungus infection and hypovirus infection, suggesting that, in the long run, hypovirus infection decreases the ability of the American chestnut population to persist.
- The reappearance of large reproducing chestnut trees, associated with a large proportion of *C. parasitica* being parasitized by hypovirus is currently taken to indicate recovery of American chestnut populations.
- However, our analysis indicates that, in the long-term, hypovirus infection hinders the ability of the American chestnut population to persist.
- What gives?

Population inertia going from diseased to recovered:

- Transient dynamics of populations perturbed from their long-term stage distribution (via infection or recovery, for example) result in a phenomenon called population inertia p_∞ (Koons, Holmes and Grand 2007, Stott et al. 2012).
- For linear models, population inertia indicates how much of a boom ($p_\infty > 1$) or bust ($p_\infty < 1$) is elicited by a non-stable population distribution $v_{t0} = n_{t0} (\|n_{t0}\|_1)^{-1}$ at time t_0, relative to that achieved by the stable population distribution v.

- In other words, p_∞ is a measure of the persistency and direction of transient dynamics on population density.
- For the model in Davelos and Jarosz, p_∞ = 0:73 when transitioning from healthy to diseased, but p_∞ = 1:63 when transitioning from diseased to recovered.
- 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 that the population with boost in the short run.
- Future research will need to determine if there are additional conservation measures that, in addition to increasing population inertia p_∞ can lower the stability radius p_e and hence increase the chance of long-term population persistence.

Matthew Kolp, Michigan State University

The role of secondary fungi in controlling blight within cankers. Between 2001-2007, a trend began to emerge at the West Salem, WI chestnut stand in that there was an increase in the recovery of other fungi (secondary fungi) isolated from chestnut cankers. By 2007, secondary fungi accounted for more than half of the isolates recovered. Kolp posed the following questions:

- What is the pattern of secondary fungi in natural cankers?
    - Are they similar across chestnut populations?
    - Are they associated with girdling or non-girdling cankers?
    - Are they associated with cankers treated with hypovirulent strains?
- Can secondary fungi influence *C. parasitica* growth and canker expansion?

In order to assess how species of secondary fungi are distributed across chestnut populations, Kolp is using the following methods:

- Ten populations will be examined: West Salem, WI; five populations in MI (both recovering and non-recovering sites); four populations from the eastern U.S.
- 12 samples/canker taken around the canker margin
- Surface sterilization of bark plugs, plated onto PDA to extract DNA from each culture
- Sequence of ITS region
- Pairwise sequence alignment with BLAST and MycoBank
- Morphological characteristics will be used to confirm fungal identity

In 2013, 208 cankers were sampled among the 10 sites for a total of about 2500 bark plugs. This assessment underway.

In 2012, 89 cankers were sampled (48 from West Salem, WI; 21 from Roscommon County, MI; and 20 from Manistee County (County Line), MI. The following table details some of Kolp's findings.

Bark isolates	West Salem, WI	Roscommon County	Manistee County
% *C. parasitica*	78.5%	92.8%	76.4%

% Non *C. parasitica*	21.5%	7.2%	23.6%
% *Trichoderma* of Non *C. parasitica*	56.5%	11.1%	5.5%

	West Salem, WI	**Roscommon County**	**Manistee County**
Girdling Cankers	**% of total isolates (Canker #)**	**% of total isolates (Canker #)**	**% of total isolates (Canker #)**
C. parasitica isolates	90.2% (22 cankers)	93% (11 cankers)	78.3% (10 cankers)
Non C. p. isolates	9.8%	7%	21.7%
Non-girdling cankers			
C. parasitica isolates	68.6% (26 cankers)	92.5% (10 cankers)	74.6% (10 cankers)
Non C. p. isolates	31.4%	7.5%	25.4%

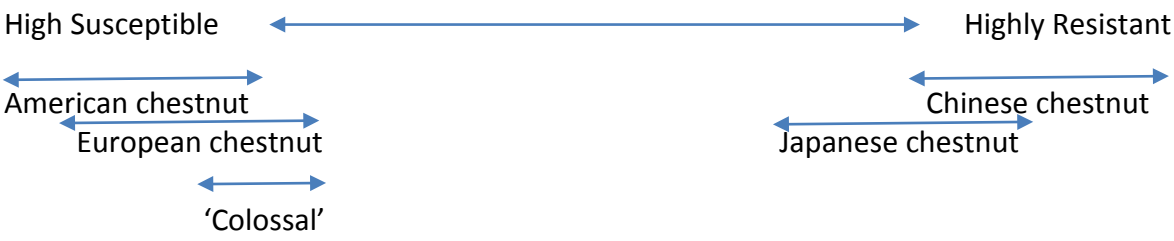
Kolp raised the questions, (1) Can secondary fungi influence *C. parasitica* growth and canker expansion? (2) Do secondary fungi influence canker expansion on live trees? and (3) Is hypovirulence important for this effect?

To see if secondary fungi influence *C. parasitica* growth *in vitro*, Kolp is co-inoculating *C. parasitica* and secondary fungi on PDA and Endothia complete medium. Species of *Trichoderma, Umbellopsis, Paraconiothyrium, Siricoccus,* and *Penicillium* were tested. The *Trichoderma* spp. had the greatest inhibition of *C. parasitica*.

To answer the question if secondary fungi influence canker expansion on chestnut tissue, Kolp will examine excised stems treated with secondary fungi that will be subsequently inoculated with *C. parasitica*. The same experiment will be conducted on living stems. He is also exposing small branch cuttings to secondary fungi for 2 weeks after which time they will be autoclaved and *C. parasitica* will be inoculated to assess the growth responses of *C. parasitica*. Kolp also will initiate *C. parasitica* cankers in living trees after which time the cankers will be challenged with secondary fungi and/or hypovirulent isolates to address the question is secondary fungi alone are capable of controlling canker expansion or if hypovirulence is necessary.

Josh Springer, Michigan State University

Hypovirulence and the biological control of chestnut blight in Michigan orchards. Hypovirulence works well in many areas in Europe and in Michigan but it does not work in the eastern U.S. We have difference in susceptibility among chestnut species. There are natural chestnut populations in MI and many have been infected for a long time. Recovering chestnuts were found in MI in the early 1980s. Some naturalized chestnut populations remained uninfected until the mid-to-late 1990s. Swollen cankers do not always indicated recovery. Can we use native hypoviruses found in MI to manage hybrid chestnut orchards in MI? The chestnut industry in MI is growing. Many of the orchards are planted with Japanese x European hybrid 'Colossal' that is mildly susceptible to chestnut blight. Can this mild susceptibility be combined with a native hypovirus to manage chestnut blight and maintain nut production? Springer provided the following chart on the differences in susceptibility among chestnut species and hybrids.



In treating orchard trees with hypovirus, Springer detailed work at the MSU East farm where 'Colossal' was planted in 1997 as bare root, budded trees. Chestnut blight has been an ongoing problem in the orchard. Springer used eight disease-free trees, ten tree with natural cankers that were treated with hypovirus and ten trees that were blight infected and left untreated. He added that the natural infections were several years old and as a result, some cankers were quite large. The blight cankers on the 'Colossal' trees had collapsed tissue, cracked bark and wilted leaves. Inoculations were made in May 2011, May/June 2012 and May 2013 (only when necessary) using the 'scatch' method applying a hypovirus slurry. The cankers were covered for 48 hours-to-one week to retain moisture and allow the inoculum to spread hypovirus to the canker thallus. The goals were to prevent dieback of the main stem, maintain tree growth and nut production. Early observations included:

- All 8 disease-free trees have survived.
- All 10-hypovirus treated trees have survived.
- 9 of 10 blight infected trees have survived.

The 2013 results are:

- Some disease-free trees are now infected
- Some trees have been retreated
- Blight-infected (non-treated) trees continue to die

Treatment data is shown in the following tables.

Treatment	Avg. increase in trunk size 2001-2012	Range of increase
Disease-free	1.21 cm	0.5 to 1.9 cm
Hypovirus treated	0.93 cm	0.6 to 2.0 cm
Blight infected and non-treated	0.72 cm	0.4 to 1.3 cm

Treatment	Nut harvest 2011 (pounds per trees)	Nut Harvest 2012 (pounds per tree)	% Change 2011-2012
Disease-free	55.7	71.1	+ 28%
Hypovirus treated	45.3	47.6	+ 5%
Blight infected and non-treated	41.8	30.7	- 27%

For 2013, they will sample infected commercial orchards and identify the *C. parasitica* vegetative compatibility (vc) diversity within each orchard. Hypovirus has already been introduced into each orchard. Inoculating takes a long time as the isolate from each canker has to be independently infected with a hypovirus and this takes time and repeated attempts in the laboratory. Some MI orchards share the same vc types so hypoviruses can be shared among some orchards. The vc diversity among seven MI orchards ranges from 1-to-5 vc types per orchard.

In terms of 'Colossal' trees, 70% of the cankers are found on the south or southwestern area of the tree (at the base) while 30% of the cankers are found near the first branch point.

With regard to hypovirus inoculum, it may need to be applied repeatedly. Spread within a tree occurs more readily than tree-to-tree spread. New cankers may appear faster than the spread of hypovirus. Untreated orchard tress will die eventually while some cankers may only be treated once to achieve 'healing'.

Chestnut blight is established in the MI orchards but it can be managed with hypovirus biological control. It is also possible to switch to cultivars that are even less susceptible than 'Colossal'. A possible choice would be 'Bouche de Betizac'.

Do mycoviruses alter population structure of the chestnut blight pathogen? Hypovirus effects and consequences:

- Effects
    - Reduce pathogen growth
    - Inhibit sexual reproduction
        - Reduces asexual sporulation
- Predicted consequences to pathogen
    - Reduce virulence on tree host
    - Change population structure—more clonal

Michigan chestnut populations are a patchwork of populations.

- Epidemic sites (no hypovirus)—highly virulent pathogen with sexual recombination and ascospore production
    - Leelanau
    - Stivers
    - Missaukee Diseased
    - Missaukee Healthy
- Recovering sites (caused by hypovirulence) have a lower pathogen virulence with asexual reproduction (no recombination) and conidial production
    - Frankfort
    - County Line
    - Roscommon

Predictions of blight pathogen population structure

- Long-term epidemic
    - Sexual (recombination)
        - Ascospores produced; long-distance wind dispersed
    - Higher genetic diversity
- New Infected

    - No a priori predictions
- Recovering
    - Asexual
        - Conidia produced; short distance dispersal
    - Low genetic diversity

The diversity within the chestnut blight pathogen is measured in two ways: (1) phenotypic level via vc; and (2) genotypic level by microsatellite diversity. The diversity in *C. parasitica* is detected using vc assays. Barrage zones form when mismatches occur at loci controlling anastomosis. This is strong frequency-dependent selection. Vegetative incompatibility helps isolates escape infection from alien nuclei, mycoviruses and defective mitochondria.

VC diversity summary

	1996			2009		
	# vc groups	**Shannon**	**Reproductive mode**	**# vc groups**	**Shannon**	**Reproductive mode**
Epidemic	14	1.64		12	1.4	
Stivers	9	2.01	Sexual	11	1.95	Sexual
Miss. Dis.	6	1.27	Sexual	6	1.09	Sexual
Miss. Healthy	ND	ND	ND	6	1.24	Asexual
Leelanau	ND	ND	ND	6	1.33	Asexual
Recovering	8	0.29		11	0.56	
County Line	2	0.09	Asexual	2	.14	Asexual
Roscommon	2	0.09	Asexual	1	0.00	Asexual
Frankfort	4	0.70	Sexual	8	1.53	Sexual

What is happening at Frankfort? The number of vc groups increased from 4 in 1996 to 8 in 2009. There is nearly a 50:50 mating type ratio and the hypvirus incidence from >90% in 1996 to 76% in 2009. The Shannon index more than doubled from 1996 to 2009. Recent mortality rates at Frankfort are higher than at Roscommon and County Line. Springer questions if there has been a change in the environment. Hypoviruses can invade but they also can be lost.

How is the underlying genome of *C. parasitica* organized? Does the pattern of microsatellite diversity mimic vc diversity? Microsatellite markers were developed for European *C. parasitica* populations. Springer used the same isolates from the 2009 vc diversity analysis for genomic diversity analysis. Ten of thirteen European markers could be amplified. Nine loci provided good information for analysis. Six of seven populations from the vc study could be used for microsatellite analysis. Missaukee Healthy had poor amplification across all loci. AMOVA results were: among population variation = 15% $\phi PT=0.147$, $p<0.001$; and within population variation = 85%.

Pairwise population differentiation (ϕPT)

	MD	**ST**	**LE**	**CL**	**FR**
MD	--				
ST	0.117	--			
LE	0.029 n.s.	0.092	--		

CL	0.104	0.194	0.185	--	
FR	0.107	0.155	0.050	0.214	--
RC	0.172	0.173	0.124	0.275	0.170

Indirect measure of gene flow between populations. Higher values here indicate that two populations are more divergent from each other genetically while lower values will then indicate that two populations are less divergent genetically.

Within population variation for microsatellites

Diversity	Population	Shannon Diversity	Avg. # Alleles/Locus	% Loci Polymorphic
High	MD	0.37 (0.11)	2.00 (0.26)	70
Low	ST	0.21 (0.09)	1.50 (0.22)	40
High	LE	0.45 (0.11)	1.90 (0.23)	70
Low	CL	0.16 (0.09)	1.40 (0.22)	30
High	FR	0.41 (0.13)	1.88 (0.29)	63
Low	RC	0.26 (0.11)	1.40 (0.16)	40

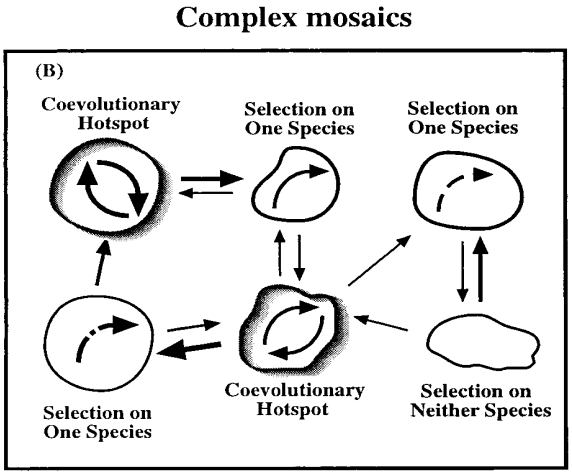
Shannon diversity close to zero indicates lower diversity in a population, that is, there's lower probability of seeing a new allele in a random sample. Average number of effective alleles for epidemic sites was generally higher than the number of effective alleles for recovering populations. And the number of loci that were polymorphic for each site also generally fit this pattern. However, Frankfort is not fitting the mold of a recovering population and neither is Stivers.

Why do blight populations have different dynamics at different sites? Hypoviruses are present in some populations of the fungus. Each recovering site has a different hypovirus type and each were most likely independent colonization events. Selection may have been able to sort through vc/mycovirus combinations.

Michigan is a patchwork of populations

- Hot spots of coevolution with pathogen
    - Plant-pathogen coevolution
- Hot spots where hypoviruses are present
    - Tri-trophic coevolution
- Dynamics can change (Frankfort)

Springer then referenced John Thompson's specific hypotheses on the geographic mosaic of coevolution (1999).



Where there's no coevolution because there's only the tree. Then one where blight is present. Then one with all three are present. Arrows are pathogen migrants.

In Michigan, pre-1930s chestnut populations were healthy. In the early 1930s, chestnut blight was detected in five of the seven sites. From the 1930s to the 1980s, there was a lot of gene flow among the MI chestnut populations with sexual reproduction. In the 1980s, there were independent colonization events of hypovirulent isolates. From 1980s to 1996, there was tritrophic (three species) coevolution (host tree, host fungus and hypoviruses) at three of the sites (CL, FR, RC). After 1996, two more sites became infected (MD and MH) and Frankfort began to lose hypovirus. Thompson's geographic mosaic theory also states that the environmental conditions can be very different at each of the MI chestnut populations generating very different dynamics.

Sandra Anagnostakis, The Connecticut Agricultural Experiment Station

Biocontrol virus maintained in American chestnut trees. In 1976, 71 three-year-old American chestnut trees were planted at the Experiment Station farm in Hamden, CT. The seed was from Michigan and Wisconsin and had been raised by Eyvind Thor (University of Tennessee). Chestnut blight cankers developed naturally on the trees. Starting in 1978, every canker that could be reached from the ground was treated with a mixture (slurry) of eight hypovirulent strains[1] grown on Potato Dextrose Agar (Difco) and mixed in a blender. The slurry was placed in 1 cm diam. holes made around the canker margins with a cork-borer through the bark to the cambium. The filled holes were then covered with paper masking tape to prevent drying. The hypoviruses in these strains were from Italy[2], France[3], and Michigan[4]. Treatments with the mixture of strains were done from 1978 through 1981 as cankers were found.

In 2013, the trees were 40 years old, and half of them continue to die back to the base from chestnut blight, sprout, and die back again. The rest continue to grow, with heavily callused cankers from the base to near the top. Branches die and new cankers form, but the trees survive and nut production is heavy. There is no pattern to the location of the "good" trees in this planting (Fig. 1), nor any obvious difference in the cankers. It is clear that all American chestnut trees are not equal in resistance to chestnut blight disease. In November

2012, the trees in the eastern row were examined and 15 of the 18 trees had live sprouts with new cankers. Bark samples were removed from the margins of new cankers and put in a freezer for three days to kill any insects present. The samples were then placed onto 2% water agar. When mycelium emerged, 57 isolates of *C. parasitica* from 14 of the 15 trees were transferred to PDA. These isolates were transferred to PDA plates overlaid with sterilized cellophane. When the cultures were about 6 cm in diameter, mycelium was scrapped from the cellophane and frozen. Mycelium was extracted with an RNeasy Plant Mini Kit (Quiagen), and extracts and ladder solution were placed in wells of 0.8% agarose E-GELs (Invitrogen) for electrophoresis in an E-Gel iBase (Invitrogen). Gels were examined and photographed, and 42 of the 57 isolates had dsRNA. Only one of the cankers failed to yield isolates with dsRNA.

[1] ATCC #38767, 38769, 38758, 38759, 38760, 38756, 38757, 38765
[2] Jean Grente, near Marseille, Les Mayons var. France
[3] Lorenzo Mittempergher, Firenze, Italy
[4] Mrs. R. D. Johnson, Rockford, Michigan

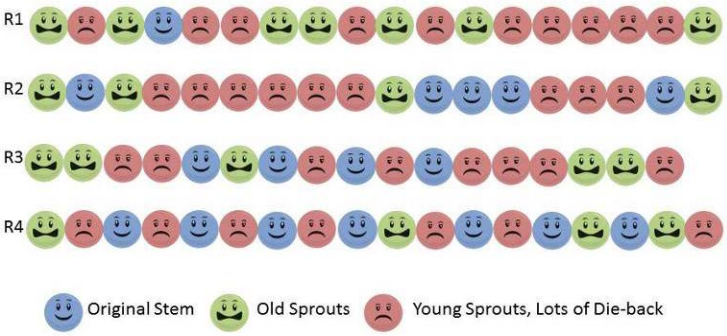


Figure 1. Condition of 40 year-old American chestnut trees at the CAES farm in Hamden, CT in 2013.

Claire Moore, Michigan State University

Diversity of vegetative compatibility genotypes in recovering and epidemic populations of *C. parasitica* across MI. There is a great deal of variability in the success of hypovirus in *Castanea* spp. around the world. Why is there recovery at some sites and not others? One hypothesis is that it may be the number of vegetative compatibility groups (vcg) in a population. Any mismatches at vegetative incompatibility (vic) loci can further inhibit hypovirus spread. There is a range of variability in effectiveness of loci (*vic 1-4, 6, 7*) at excluding invaders. She wants to see what variability there is in MI populations. In epidemic populations, there is a higher rate of sexual reproduction and, consequently, more diverse vcgs. In recovering populations it is hypothesized that the variability occurs only at 'weak' vic loci and/or the strong loci are fixed. Hypovirus spread would increase in a population with an increase in variability at weak loci. Moore posted three questions:

1. What is the genotypic diversity of vic loci within and between MI populations?
2. Do recovering and epidemic populations differ in variability at vic loci?
3. Are the 'weak' loci more diverse in recovering populations than in epidemic populations?

Her three goals are:

1. Identify patterns of vic loci diversity within and among populations
2. Characterize vic loci responsible for vcg diversity
3. Analyze allelic diversity and geographic distance between populations

Moore's study sites are:

Recovering Populations
Hypovirus: high frequencies
2009—11 vcgs
County Lin (CL)
Frankfort (FR)
Roscommon (RC)

Epidemic Populations
Hypovirus: low frequencies
2009: 19 VCGS
Leelanau (LE)
Missaukee Diseased (MD)
Missaukee Healthy (MH)
Stivers (ST)

Moore conducts vic genotype identification using 64 European Union testers and 18 MI vcgs. Tests are conducted on PDA embedded with debarked, autoclaved American chestnut wood. Pairings are replicated three times and the barrage line formation (or lack thereof) are read at 2-3 weeks. When results are ambiguous, four additional replicates are conducted.

Results of the vic genotypes in MI

Michigan VC Group	EU VC Tester:	Assigned *vic* genotype	*vic locus*					
			vic1	*vic2*	*vic3*	*vic4*	*vic6*	*vic7*
1	17	2112-11	2	1	1	2	1	1
2	28	2212-11	2	2	1	2	1	1
3	13	1211-11	1	2	1	1	1	1
4	12	1112-11	1	1	1	2	1	1
5	40	1122-11	1	1	2	2	1	1
9	11	1212-11	1	2	1	2	1	1
10	15	2211-11	2	2	1	1	1	1
12	33	2222-11	2	2	2	2	1	1
13	45	2121-21	2	1	2	1	2	1
14	9	2111-11	2	1	1	1	1	1
15	30	1212-12	1	2	1	2	1	2
16	5	2211-22	2	2	1	1	2	2
17	1	2212-22	2	2	1	2	2	2
FR65, CLD51, RC242, FR449, ST192: NO MATCH								

There is a lot of variability in vic 1-4 and much less variability in vic 6 and 7. There were 5 isolates for which she had no match; she did not include the Roscommon site in this study as none of the isolates from that site matched any of the 64 EU testers. Vic 6 and 7 are fixed for most of the sites. Vic 1 and 2 are variable at most locations except for CL where it appears they are fixed. Within populations, in terms of variability, was not what she expected.

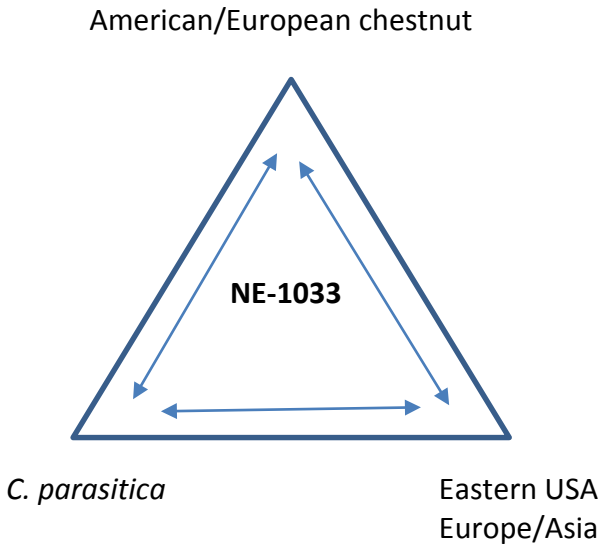
There is only a weak relationship between genetic distance and geographic distance. Further analysis and more data are needed.

Conclusions:

In the statewide analysis, 13 of 18 MI vcgs were identified. The isolates that did not match with any of the 64 EU testers indicates the possibility of an uncharacterized vic locus. Overall, there is variability at vic loci 1,2,3,4 but not at vic 6 and 7. Vic loci 1 and 2 were the most variable (note that CL was fixed at all six loci). The epidemic locations are variable at all six loci while the recovering populations are variable at four vic loci. Contrary to her expectations, the recovering sites are variable at two of three vic loci thought to inhibit hypovirus spread (vic 2 and 6). Thus, vegetative compatibility may not have as much control over hypovirus spread as was originally thought.

Dennis Fulbright, Michigan State University

Odd cankers in Maryland. Fulbright took a trip to Maryland and every now and again, and, as he state, 'some things change your perspective on chestnut'. He has had three changes in the thirty years that he has worked on chestnut. One of those was the cankers on irradiated trees in Maryland.



The evolution of his last 'change' was Marlene Haggblade's study on the Maryland cruddy bark trees. We each work in one corner of the above triangle but we don't have an overall understanding of the interactions. The USDA regional project was designed to put together an outcome—that is a tree resistant to chestnut blight.

In Maryland (with Gary Carver), Fulbright expected to see small trees with really large cankers that Hebard called "big ugly", but he saw large trees with strange cankers—multiple infections, almost systemic in nature. Many cankers had the signature of hypovirulence. The trees were very isolated—some trees had some mature bark that was beginning. Some of the trees had cruddy bark, others normal bark. A lot of samples were taken but it was difficult to try and find *C. parasitica* stroma. The trees were in varying stages of health—some had good canopies, other were thin. Most of the time, the sprouts were dead. There was some swelling on a few of the living sprouts. Hardly any sprouts were as healthy as the mother tree. Some surviving trees had no cruddy bark at all. Some of the trees are European-looking—stout branches with clusters of burs, typical of European chestnut.

He also visited a row ACCF trees (Gary and Lucille Griffin) at Tom Sciviner's location. Most of trees looked good but some of the dead trees had been cut. These trees had some dead branches. Some of the trees had thick leaves. Leaves looked different from tree-to-tree. Fulbright questioned what we have learned in the past thirty year. We should be able to take material from a tree and say whether the tree has changed, the fungus has changed… Are there other organisms responsible? This is a phenomenon in a tight area of the eastern U.S. (MD, VA)—these trees may have a different level of resistance. His hypothesis is that in the 1960s when chestnuts were irradiated, tens of thousands of irradiated nuts were planted. We know where some of them were planted but we don't know where all of them were planted. There had to be a lot of nuts that volunteers walked away with. Craddock noted that he sees similar things in TN and he too believes that it is a host response. In the 1750s, the DuPont's were growing groves of European chestnut. Thomas Jefferson planted European chestnuts that he imported from France. Thus, European chestnut has been grown in the U.S. for 200 years. Fulbright cannot see the commonality among trees planted in state parks in VA and trees from Cadillac, MI. Anagnostakis commented that boxcar loads of 'Paragon' trees (American X European hybrids) were planted all across the country. Hebard commented that American chestnut produced enormous amounts (billions) of chestnuts and there should be some expected frequency of resistance (1 in a million/billion) in the population. A lot of the trees in MD were released by gypsy moth defoliation and many of the trees that Hebard and Griffin sampled in 1980 had *C. parasitica* isolates with reduced virulence. Before the release, the tree population was very low with very low levels of fungus. Now that the trees are larger, hypoviruses are emerging. There is also introgression with Chinese and European chestnut. Van Alfen added that there may be an evolution to lower virulence in the fungal population gradually occurring due to the generation times of the fungus. Maybe these lower virulent isolates can kill sprouts but larger trees and trees with increased resistance are not killed. Fulbright would like to core many of these trees with odd cankers to see if they were released at the same time or if they were planted as opposed to trees that were released from a canopy disturbance. There also may be other fungi involved. Anagnostakis stated that the most common 'contaminant' in CT trees is *Pestalotia* while it seems to be *Trichoderma* in other areas. Kaczmeirczak commented that they often have to re-treat cankers as the hypovirus seems to be lost. Fulbright cited work by Daniel Rigling in Switzerland who found that hypoviruses decreased over time in European chestnut using the argument that hypoviruses need a living host (fungus) and once the fungus declines, so does the hypovirus. In the best recovering sites in Italy, 85% of the cankers had hypovirus. Twenty years later, that figure was still 85%. Fulbright closed his session by stating that you cannot look at the MD trees and state, 'this is hypovirulence', as the cause of the odd-looking cankers is not always evident. If the cause is found to be irradiation, then that had to be transmitted to the seed because of the similar response (swelling) of non-hypovirulent irradiated trees in MI.

<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

Ignazio Graziosi, University of Kentucky

An opportunistic fungus causes mortality of the Asian chestnut gall wasp. Due to Phytophthora root rot (1800s) and chestnut blight (early 1900s), the American chestnut has been functionally eliminated from the eastern N.A. forests. Additional current pests of chestnut include the Asian chestnut gall wasp, ambrosia beetles and Japanese beetles.

The invasive Asian chestnut gall wasp (ACGW), *Dryocosmus kuirphilus*, a cynipid wasp, is a global pest of chestnut. It is monophagous on *Castanea* with one generation per year. Production is asexual (females lay eggs without mating) and this pest results in tree decline and yield loss. In the spring, galls appear on shoots and inside the larvae feed on host tissue. The wasps emerge in mid-summer. The adult wasp deposits eggs in chestnut buds; the first instar larvae remain in the buds all winter. Graziosi focused the remainder of his talk on the stage when the larvae are inside the gall (spring-to-early summer). This is the period when natural enemies are active. The most important enemies of ACGW are parasitoid wasps, introduced from China for biological control. Parasitoid wasps lay eggs inside the gall and the parasitoid larvae feed on the ACGW larvae. The parasitoid larvae overwinter in the galls and emerge in the spring.

Necrotic lesions on galls, associated with gall wasp mortality, has been observed consistently in N.A. There is an interaction between the microorganism, the tree and insect and he wonders what impact this microorganism has on the parasitoids. Graziosi's goal was to identify the organism associated with gall mortality and evaluate the impact on insect survivorship. To do that, he collected symptomatic galls from two Chinese chestnut trees in Fayette County, KY. He made three collections: (1) early (larval development); (2) intermediate (pupal development); and (3) late (after adult emergence) to follow infection and the development of the insects inside the galls. He dissected the galls and isolated from the galls and insects inside the galls. He used the isolations to perform artificial inoculations in the field and in the lab. During his dissection, he found both living and dead gall wasps and necrotic tissue surrounding the gall chambers. He also detected mycelium in the galls and parasitoid wasps. Some of his data is in the following table (small sample size, 23 total galls).

	Early (31 May)	Intermediate (8 June)	Late (17 July)
% necrotic chamber	0%	55%	66%
% dead gall wasp	0%	100%	36%
% parasitization	15%	45%	35%
% dead parasitoid	0%	11%	3%

The fungus was isolated from necrotic galls and he used this isolate to perform Koch's postulates. Eighty-eight percent of the necrotic galls yielded a "pink" pigmented fungus. The pink fungus was isolates from 92% of insect cadavers. In the field, Graziosi made artificial 2-mm wounds, using three fungal strains and an agar control, by inserting a disc of the fungal culture. Five Chinese chestnut trees were used, three branches per tree and four galls per branch. Infection was assessed after two weeks. He evaluated the size of the necrotic area (sq. mm). Necrosis was much greater with the fungal isolates than the agar control. He also assessed gall wasp mortality and he reisolated the fungus. The galls were 100% symptomatic and no living ACGW were found. There was virtually no parasitoid mortality. In the lab, he used one fungal strain and an agar control. The chestnut seedlings were grown in a growth

chamber and he inoculated one gall per seedling with five total seedlings. The necrotic lesion from the fungus averaged 90 sq mm while the control was less than 15. He was able to reisolate the same fungus and he identified the fungus as *Colletotrichum* through GeneBank. This same fungus has been isolated from ACGW in Japan. *Colletotrichum/Glomerella* sp. is an anthracnose disease of fruit trees. The fungus colonizes in the late spring. After superficial lesions, the fungus rapidly penetrates the gall tissue inside the gall chamber. There is high gall wasp mortality and no parasitoid mortality. This is a plant pathogen and an opportunistic entomopathogen.

Sandra Anagnostakis, The Connecticut Agricultural Experiment Station

Plantings of bare-root seedlings and containerized seedlings (with Scott Schlarbaum and Cornelia Pinchot). Open pollinated seed (2010) from a Japanese chestnut, an American chestnut and three hybrids was grown in the East TN State Nursery, and in containers by the Forrest Keeling Nursery in MO. Dormant bare root trees from TN and their half-sibs in containers were planted in three sites in CT in early spring 2012. Tree tubes protected the plants from predation. In the fall of 2012, the seedlings were measured and survival tallied:

<u>Griswold</u>

Bare-root	107/109 alive (98%) with 40 cm. average height growth
Containerized:	84/109 alive (77%) with 11 cm. average height growth

<u>Sprague</u>

Bare-root	46/60 alive (77%) with 36 cm. average height growth
Containerized:	40/60 alive (67%) with 25 cm. average height growth

<u>Goshen</u>

Bare-root	21/24 alive (88%) with 15 cm. average height growth
Containerized:	13/24 alive (54%) with 22 cm. average height growth

After measuring again in the fall of 2013, Pinchot will compare family results using statistical analysis.

Plantings of bare-root chestnut trees and direct seeding (with Cornelia Pinchot). Open pollinated seed from a Japanese chestnut, and two hybrids was collected in 2012, and stored in damp sphagnum at 32°C. In early March 2013, half of the seed was planted in the CAES greenhouse. The other half of the collected seed was kept in damp sphagnum at 32°C. The first week of June 2013, alternating seed/bare-root seedlings of the same half-sibs were planted at the CAES farms in Hamden and Griswold, and on private land in Warren, CT. Tree tubes protected the seed and plants from predation. In August 2013, we noted which had survived:

<u>Hamden</u>

seed	42/46 alive (91%)
seedlings	42/46 alive (91%)

<u>Griswold</u>

seed	29/52 alive (56%)
seedlings	49/49 alive (100%)

<u>Warren</u>

seed	17/24 alive (71%)

seedlings 24/24 alive (100%)

After measuring height growth and noting survival in the fall of 2013, Pinchot will compare family results using statistical analysis.

'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. These trees are available for future genetic studies.

Michael McCampbell, Frostburg State University

Seedling establishment of American chestnut in PA. McCampbell is interested in how chestnut seedlings will perform in the field. Can seedling performance be related to any particular variable and what are the best protocols to promote growth and survival? McCampbell used pure American blight-susceptible chestnut to assess the influence of site factors on survival and growth. He planted on four sites PA. He used 12 Op families and measured height, root collar diameter (rcd) and first-order later roots >1mm (folr).

Successful planting begins with high quality seedlings. Seeds were collected from 12 trees in PA in 1998, planted at the Flint Creek nursery in GA. Paul Kormanik, who is retired from the USDA Forest Service and others, developed unique nursery protocols that develop a strong root base so that seedlings will sprout when browsed and get above deer browse as soon as possible. After they were lifted from the nursery, the seedlings were shipped to UT where Scott Shlarbaum's crew took measurements (ht, rcd and folr) in 1999. Chestnut has a much more fibrous root system compared to oak.

The four PA sites were: (1) Buchanan State Forest, a dry site; (2) Clear Creek State Forest—this site was clearcut in 2007 and was removed from the study; (3) Delaware State Forest; and (4) Sproul State Forest. The sites were all fenced to minimize deer browse. Most of his data is from the Sproul and Delaware sites as the Buchanan site was very dry and there was <2% survival of chestnut; there was >50% mortality after the first year. After 13 years, there is blight on about 70% of the survivors. The largest chestnuts are 9.8m in ht and 13 cm dbh. New seedlings are growing at the Delaware site. There was 71% survival after 13 years at the Sproul site and about 30% at the Delaware site. McCampbell showed data on survival based on initial ht. rcd and folr. The greatest impact was seen by folr as the seedlings with 15-30 folr had a survival rate of 70% compared to seedlings with 0-5 folr whose survival was less than 40%. The impacts of ht, rcd and folr were significant for the first years in terms of survival and time to height exceeding deer browse. The ideal seedling for hands-off restoration: >99 cm in ht; >6 mm rcd; and >5 folr.

Stacy Clark, USDA-Forest Service, Knoxville

2013 University of Tennessee chestnut research summary. Schlarbaum at UT uses his existing infrastructure to play a supportive or co-leader role with cooperators because of other priorities in Tennessee, mainly with timber species. Schlarbaum's other activities include:

- Evaluation of American chestnut genetic families planted at four locations in PA (covered by Michale McCampell at Frostburg)
- Testing American chestnut Foundation BC_3F_3 seedlings in silviculture tests (in conjunction with Stacy Clark)
- Contrast bare-root seedlings with Forrest Keeling Nursery containerized stock (covered by Sandra Anaganostakis in association with Leila Pinchot)
- Testing American Chestnut Cooperators Foundation VT1 seedling (in conjunction with Lucille and Gary Griffin)
- Evaluation of Asiatic oak weevil damage on American chestnut and TACF BC_3F_3 seedlings in siliviculture test (in conjunction with Stacy Clark and Bud Mayfield—USFS)—Ashley Case, a graduate student working in one of Clark's TN sites found that Asiatic oak weevils were least abundant on Chinese chestnut.

American chestnut restoration on national forests in the southern region. The first test plantings by the USDA Forest Service, The University of Tennessee and The American Chestnut Foundation—the goal was: To test TACF American chestnuts that have been traditionally bred for blight-resistance for the ability to survive, compete and remain blight-resistant in forests within the species' native range. The nursery production was as follows:

- bare-root nursery stock used in 2009, 2010, 2011 plantings
- Seedling size maximized using Paul Kormanik's work
- Split seedlings into two size classes
- Averaged 1m for 2009 plantings; 1.3m for 2010 and 2011 plantings

The field plantings:

- Seedlings were planted on newly harvested sties (shelterwood with reserves, $3 m^2 ha^{-1}$ basal area)
- Stump sprouts were chemically treated—worked well for all species except red maple
- Site index ~26 (Northern red oak, base age 50)
- It is just as important to determine if these trees can adapt in this environment as it will be to determine blight resistance.
- 11 plantings were established in 2009, 2010 and 2011 in TN, VA and NC
    - Planted 4596 trees: a mixture of American (837), Chinese (535), BC_1F_3 (470), BC_2F_3 (455), BC_3F_2 (277) and BC_3F_3 (2022)
    - Incomplete block design, single tree plots were used
- Treatments tested
    - Location
    - Generation/Parental species
    - Family (Generation/Parental species)
    - Seedling size (large vs small)
    - Year
    - Interactions

Data collection was as follows:

- Before or at planting: Nursery seedling measurements
    - Height, root-collar diameter, number of first-order lateral roots (FOLR)
- Sprouting

- Bud-break phenology
    - Ranking assigned when bud-break is underway
        - 0=not broken
        - 5.9=90-100% of tree is fully leafed out; 0.10 increments)
- Deer browse to terminal bud
- Stem dieback and sprouting
- Blight
    - Blight recorded on live trees (conservative identifications, some cultured to confirm ID)
- After bud set: Height, ground-line diameter
- Competition in 1.3 m radius plot around tree
    - Tallest competitor, most abundant competitor
- Also note insects, defoliation, other conditions

Fourth year height data from the 2009 plantings—total height was 2.2m across all sites, ranging from 0.-2m to 5.3m. The large size-class trees were 0.4m taller than the small size-class trees—bottom line is bigger seedlings become bigger trees. American chestnut was significantly taller than any of the BC lines and they were significantly taller than the Chinese chestnut. The mean survival after four years was 77% (large-73% and small 80%). In terms of cankers, all BC_3F_3 families were the same and equal to Chinese chestnut and it is too early to make inferences regarding resistance. With respect to deer browse, a 0.5m trees at planting was five times more likely to be browsed than a 1.5m tree. Deer seem to be selecting American and BC_3F_3 over Chinese and the other BC trees. In terms of bud break phenology, it is strongly related to generation in that the BC_3 break bud more quickly than Americans and this was significant across all locations.

Phytophthora was primary cause of mortality in the 2011 plantings—Differences in mortality was mostly related to site conditions. The fungus probably came from the nursery. As a result, her recommendation is to stop using bare-root seedlings in the south because of *P. cinnamomi*.

Preliminary conclusions:

- *Phytophthora* and deer were major barriers to early restoration success
- Larger trees had better total height after 4 years, but had 6% lower survival compared to small trees
- Chestnut grows fast! > 1 foot/year
- BC_3F_3 not behaving exactly like Americans in height or in budbreak phenology
    - Height difference may be due to one family that is poor performer
    - Bud-break strongly related to breeding generation

The bottom line is that there is a lot more going on in the forests than just chestnut blight. Tree are also impacted by: Asiatic gall wasp; Asiatic oak weevil; aphid curling; Phytophthora root rot; chestnut sawfly; periodical cicada; and Ambrosia beetles. The bottom line is bigger seedlings become bigger trees. American chestnut was significantly taller than any of the BC lines and they were significantly taller than the Chinese chestnut. The mean survival after four years was 77% (large-73% and small 80%). In terms of cankers, all BC_3F_3 families were the same and equal to Chinese chestnut and it is too early to make inferences regarding

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Hill Craddock, University of Tennessee, Chattanooga

Craddock is still doing back cross breeding. On their high elevation sites, chestnuts flower in mid-July so there is plenty of time to collect pollen at Meadowview. Chestnuts are dioecious and thanks to Mike French, they are taking pollen from TN to Meadowview to work on a father tree program. They have been very successful, working mostly on the 'Clapper' lines. Occasionally in TN they see large survivors—trees that have had blight for many years but survive. Isolations from bark samples indicated that cankers on these trees contain dsRNA. There may be something special about these trees on a genetic level as well. Evidence for that is that these types are trees are not seen often. About 10 years ago, crosses were made from one of these trees and in 2013 first backcrosses were made. What is special about the backcross trees is that the parents were chosen in a Phytophthora environment. The genes for blight resistance come from TN sources rather than Meadowview sources. One of the challenges that he took on was to add local sources of resistance to the breeding program at the state level. They began making lots of F_1s and these backcross trees are now available to other researchers.

Craddock finds that trees do best when direct seeded. They are establishing new backcross orchards that will be screened for blight resistance at about 5 years of age. Dead trees are rouged out to winnow down to the few best trees, based on blight resistance and a whole suite of morphological characters (8-10 traits). At this level, intercrosses are all produced by hand pollination. They now have B_3F_2 and B_4F_2 plants. Blight resistance is assessed by direct in inoculation with Ep 155 and SG strains. The most susceptible trees die—they are not measuring cankers but using a visual rating. The resistant trees look very good. Craddock uses

citizen science and without the aid of volunteers, this work would not be accomplished, especially when dealing with tens of thousands of trees.

Craddock also is working on Phytophthora sreening with Clint Neal at the Army Corp of Engineers. They are conducting trials similar to that of Joe James (in containers). The susceptible trees drop out very quickly. Within a few weeks, all the American controls are dead and the Chinese survive.

A progeny test using 25 genetic families is being conducted (25 plots for a total of 625 trees) in a tornado salvage area. Both direct seed and containerized plugs were used. He has no data to present.

Craddock presented data on the photosynthetically active radiation test that included BC_3F_3 seedlings. The hybrids are reacting more or less like American rather than Chinese. What was surprising was that, in low light levels, Chinese had more photosynthetic radiation than Americans. Quantum efficiency is a measure of the fraction of sunlight absorbed that actually gets converted into chemical energy inside the leaf. Typically, we expect that shade-adapted species use light more efficiently since it is more limiting in their environments. So, they would have a higher quantum efficiency than sun-adapted plants. The take-home message is hybrids do seem to be influenced to some degree by their Chinese ancestry in terms of quantum efficiency. But, in a shady environment like a forest understory, they have similar quantum efficiency to the American chestnut. In high light levels, reduced quantum efficiency of hybrids (and its similarity) to that of Chinese chestnut could actually be a 'good thing' by protecting hybrids from photodamage.

An important milestone was passed in TN this year in that they initiated the first seed orchard based on TN material (B_3F_2 plantings) in Knox County.

Craddock recently had a student finish a thesis on chestnut cultivars. He believes that chestnut can be grown in TN as a nut crop. There seems to be a lot of interest. There are, however, a few disadvantages (insect and disease problems) that are not found in MI; Craddock feels that TN may not be as competitive in that regard. He is very interested in the idea of using hypovirulence in orchards. They have had good luck with 'Colossal' except that it is not blight resistant.

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

Identifying potential sites/growers. Participants at 2013 "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 91 individuals. Follow-up surveys utilizing the Chestnut Chatter listserv will be sent out in late September 2013.

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 2013.

A 2011 Washington County PA test/demonstration orchard was expanded in 2012and again in 2013 to include BC1 seedlings and a small planting of BC3F3 seed. This orchard was host to a 7.22.13 meeting of 105 members of the International Walnut Growers who requested a program on American chestnut culture and restoration.

US Army Corp of Engineers - Shenango Lake planting 05. 13. 2013
Collection of local genetic materials

- Collection and processing of open-pollinated American chestnut seed for TACF and PSU.

Outreach Efforts:

- "American Chestnut Restoration" was 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. (Objective III.)*
- *"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 NE1333 participant. This fact sheet was utilized again in 2013 in 4 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 1184 subscribers throughout western PA and eastern OH of chestnut-related educational opportunities.
- Staff PA-TACF exhibit and conduct chestnut orchard tours at 2013 Penn State Ag Progress Days

Bryan Burhans, The American Chestnut Foundation, Asheville

Update on TACF. The American Chestnut Foundation is not technically a foundation like the Mellon Foundation that has a lot of invested money that they donate to causes. TACF is a typical non-profit in that it must raise money every year to fulfill its mission to restore American chestnut to eastern forests. One reason non-profits have 501c3 status is that they raise funds that it takes to fulfill the mission of the foundation. He has been on board for five years and there has been an internal overhaul, everything from database management to communication. This is all with an eye on how to better serve the mission of the foundation. There are currently 16 state chapters and they implement the mission at the state level; it is where the rubber meets the road. Some chapters are implementing seed orchards. One of the principles of the foundation is the reliance on partnerships (members of NE-1033) to do much of the work—partners conduct research that will guide the future restoration of the American chestnut. While we can get excited about reintroducing a tree back into the forest, we cannot lose sight over the fact that there is still a lot of basic research that needs to be conducted. The foundation's goal is not to produce a nice horticultural specimen for front yards but populations of trees that will self-perpetuate and last long-term.

Burhans is a believer that if you want good answers, you have to ask good questions. He asked member of NE-1033 for their questions and passed out a questionnaire asking, 'What questions still need to be addressed?' The questionnaires can be returned to Burhans either in person or via mail. He will then compile the list of questions that will help with the foundation's strategic planning. In two years, TACF will undertake a major fundraising

campaign. Donors get excited about research but they want to know outcomes, costs, etc. First, the costs and needs have to be identified. TACF has increased all metrics. The challenge is to keep pace with Meadowview's needs. The state chapter seed orchards require lots of help/money. TACF is on solid ground but how do we meet tomorrow's challenges? The fundraising goal will be $25M. The hope is to increase giving by $3.5-$4M/year.

Follow-up questions. What questions need to be asked? Ideas are needed from a strategic standpoint and determine how they can be prioritized to help with long-term goals.

- Brosi commented that artisans are a community that can be engaged as she is constantly asked for chestnut and butternut for carving. Swollen cankers might be what wood carvers desire as burled wood is in fashion. We also might want to coppice trees to provide a continual source of wood for artisans. She noted that the richest person in Frostburg is a dulcimer maker.
- Fulbright—decay resistance. What age does it take for a tree to become decay resistant? Have we ever compared the rot resistance of American chestnut to pressure treated wood? Can we develop a study to show that there is a use for chestnut timber?
- MacDonald—we need to know more about hybrid trees before we have full-scale forest plantings.
- Clark—the relationship and interaction between *C. parasitica* and *P. cinnamomi* and sites need further study. Do we plant in clearcuts or even-aged gaps or xeric sites vs mesic sites? We need an understanding of elevation and the stability of blight resistance. The ecology of chestnut, including wildlife and the role of insect pests needs further study. We need a true entomological study to see what insects chestnut supports.
- Brosi—should we intentionally plant chestnut in areas where there are endangered wildlife species. This would put a whole new twist on the merits of chestnut (i.e. Allegheny woodrat and endangered warblers).
- Craddock—the thing we don't know is how to move the breeding program into the forest. Are we bringing seedlings with *P. cinnamomi* into the forests from conventional nurseries? Will TACF run its own nurseries or farm work out to commercial enterprises? It is well known that, all things equal, bare root seedling grow better and survive better than containerized seedlings but often politics come into play. Will TACF accept second best because of the fear of Phytophthora? It is difficult to know forest outcomes when the nursery issue is unanswered. Craddock hopes TACF can become a clearing house and an archive of information. What is missing is the way to coordinate all the information from chapters as chapter breeding is cumbersome. There is a big opportunity to document information.
- Van Alfen—he is struck by advancements and knowledge of host genetics and at the same time we don't have comparable data on the fungus genetics. TACF was founded on the knowledge and vision of Charles Burnham, a wheat breeder. The success of the wheat breeding program was based on the anticipation of what the fungal population would do. We need to monitor the fungal population continually, understanding what is happening with the fungus in anticipation of what the next gene for resistance needs to be. It is a continual process. We don't know much about virulence today and that may

be the single most important factor in understanding and predicting what the outcome of resistance will be.

- Hebard—one of the most important facets of TACF is the inclusion of citizen scientists. He noted that *C. radicalis* was very prevalent on American chestnut in 1913 but he doubts that it has been isolated since *C. parasitica* was introduced. Maybe it has been replaced by *C. parasitica* but it may simply be a lack of simple observation.
- Zhebentyayeva —low frost tolerance is the only limiting factor for Phytophthora.
- MacDonald—on the best sites in the central Appalachians, you don't find chestnut anymore. Chestnut is now found on rocky ridge tops with thin soils. This raises the question as to what sites are best for chestnut.
- Clark—chestnut has to be planted on very good sites. Is there a correlation between where chestnuts were and where they are now?
- Sisco—we need to at least figure out the genetics of the three major sources of resistance, 'Nanking', 'Clapper' and 'Mahogany' and we need to look at the advanced generations and see if the genes that were in BC_1 are being carried on to BC_3. We need to figure out the goal. If the goal is survival in a forest then we need good resistance. If the goal is for something ugly to survive then maybe raising the resistance just a little may suffice.
- Jarosz—after 100 years of chestnut blight, we still don't know how *C. parasitica* infects. If you know that, then you know what pathways are influence by the host.
- Zhebentyayeva—yearly performance needs a minimum of three years evaluation. For example, in Portugal, it took researchers five years to determine if trees were resistant to Phytophthora. It is fine to see that trees survive Phytophthora after one year, but trees need to be checked an additional two years as we need very resistant advanced material.

Business Meeting

Anita Davelos Baines is the chair-elect and will host the meeting 4-6 Sept 2014 at the Radisson Hotel in La Crosse, WI. Fred Hebard agreed to be chair-elect in 2015.

Respectfully submitted,
Mark Double
West Virginia University
November 2013

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