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

Cataloochee Ranch, Maggie Valley, NC

September 17-18, 2010

Attendance:

Alabama:	Fenny Dane (Auburn University), Jimmy Maddox (TVA-retired)
California:	Pam Kazmierczak, Debora Jacob-Wilk (UC Davis)
Connecticut:	Sandra Anagnostakis—Chair-elect (Connecticut Agricultural Experiment Station)
Georgia:	Erin Coughlin (Berry College), Mariah Sappington (Mercer University)
Indiana:	Jeanne Romero-Severson (University of Notre Dame)
Kentucky:	Lynne Rieske-Kinney, Ignazio Graziosi (University of Kentucky)
Maryland:	Donald Nuss, Gil Choi, Ken Jensen, Xuemin Zhang, Shin Kasahara (University of Maryland Biotechnology Institute, Shady Grove)
Michigan:	Dennis Fulbright, Andrew Jarosz, Carmen Medina-Mora, Josh Springer (Michigan State University)
Mississippi:	Tom Kubisiak, Dana Nelson (USDA-FS-SIFG)
New Jersey:	Bradley Hillman—Administrative Advisor (Rutgers University)
New Mexico:	Angus Dawe (New Mexico State University)
New York:	William Powell, Kathleen Baier, Andrew Newhouse, Katherine D'Amico, (SUNY-ESF), John Dougherty (NY Chapter TACF®)
North Carolina:	Paul Sisco—Chair (TACF®, Asheville), Ron Sederoff (NC State), Russell Regnery (NC Chapter TACF®)
Pennsylvania:	John Carlson (Penn State University), Gary Micsky (Penn State Extension Service), Sara Fitzsimmons, (TACF®, State College)
South Carolina:	Bert Abbott, Abdelali Barakat, Eric Fang, Bode Olukolu, Meg Staton (Clemson University)
Tennessee:	Hill Craddock, Amelia Harris, Keaton Hughes, Ana Metaxas (UT Chattanooga), Scott Schlarbaum, Leila Pinchot (University of Tennessee, Tennessee Agricultural Experiment Station)
Vermont:	Kendra Gurney (TACF®, Burlington), Tom Saielli (University of Vermont)
Virginia:	Fred Hebard, William White, David Bevins (TACF® Meadowview)
West Virginia:	William MacDonald, Mark Double (West Virginia University)
Canada:	Dragan Galic (University of Guelph)

The meeting was called to order by Chairman Sisco at 9:00 am on September 17, 2010 at the Cataloochee Ranch, Maggie Valley, NC. Sisco introduced the owner of Cataloochee Ranch, Judy Coker, who provided a history of the ranch. She indicated that Cataloochee is Cherokee for 'wave upon wave'. Coker's father, Tom Alexander, a forester who dappled in tourism, opened the ranch to guests in 1938. The ranch, now more than 1,000 acres, includes a main ranch house, a lodge and individual cabins. It has been in continuous operation since 1938.

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I. Presentations

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

Fred Hebard, The American Chestnut Foundation®

Fungal virulence. Mila Wilmouth conducted a pathogenicity test using 97 *C. parasitica* isolates. These isolates, along with three checks, were inoculated onto 15 chestnut (first hybrids between Chinese and American) using a randomized complete block design (100 inoculations/tree). Hebard showed a histogram of a Gary Griffin, et al. paper (1983) that showed size differences of a number of *C. parasitica* isolates from large surviving chestnut trees; inoculations were conducted between 1979 and 1981. In these pathogenicity tests, two check isolates were used, Weakly and Cool Ridge (dsRNA containing). Cool Ridge was always slightly more pathogenic than Weakly.

When Hebard first began work at Meadowview, he tested the pathogenicity of isolates that could be useful in his backcross studies. After three years of testing, using 15 replicates, Hebard was able to separate isolates from the top of the distribution (like Ep155, highly pathogenic) from those at the bottom (SG2-3) that were mildly pathogenic. When he began screening trees for blight resistance, he used both Ep155 and SG2-3. On trees with intermediate resistance, SG2-3 gives a significantly smaller canker than does Ep155. On American chestnut, there is much less separation between SG2-3 and Ep155 than on hybrids. The 97 *C. parasitica* isolates, used by Wilmouth, were progeny from a cross between SG2-3 and Ep155. Hebard felt if these isolate were put into trees with intermediate resistance (F_1s) that the separation would be readily evident and these data could be used to try and map the location of virulence genes. Unfortunately, this was not the case. Only the extremes in the distribution could be detected. (Only eight isolates could be distinguished from the highly pathogenic isolates). He attributed this to variation in the crowns of the trees, as there was a lot of within-tree variation.

Block designs. Hebard posed the question, 'Does high mortality in chestnut plantings make blocked experimental designs more inefficient than completely randomized designs?'
Hebard stated that there is strong disagreement about whether to use completely randomized designs (CRD) or blocked designs. When mortality begins, there was a claim that incomplete block designs (IBD) would lose efficiency while the completely randomized design is more robust. Hebard used the following model for experimental design:

$$Y_{ijk} = t_k + b_j + e_i$$

Where:
t=Treatment
k= Number of treatments
b=Block
j=Number of blocks
e=Error
i=Number of experimental units
When j=1, it is a completely randomized design (CRD) and when j>1, the design is an incomplete block design until j=k (when it becomes a randomized complete block design). Error has a magnitude, which is varied randomly, according to a normal distribution.

In his experimental layout, Hebard used ten designs for any point and randomized each design ten times, for a total of 100 separate ANOVAs. He assigned mortality randomly to the experimental units. When there was no block effect, the CRD was no better than the IBD, but as soon as there was a

block effect, the IBDs were better. At low error and high block effect, the IBD is better. His conclusion was to block all experiments.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

Anagnostakis only works in clearcuts in CT—otherwise it is a waste of her time. She uses areas that have a lot of native American sprouts. She first samples cankers to discern the number of vegetative compatibility types. She converts virulent *C. parasitica* isolates using French and Italian hypovirulent isolates. By the time she is ready to plant her backcross hybrids in an area, she has hypovirulent isolates to use. She uses a slurry and treats twice a year making wounds around the canker with a scalpel.

John Carlson, Penn State University

Forest Health Initiative. This project, supported by The Forest Health Initiative (http://foresthealthinitiative.org/), is a collaborative effort to advance the role of biotechnology in addressing forest health challenges, starting with American chestnut restoration. The Chestnut genome sequencing project builds upon the results of the NSF-sponsored Genomic Tool Development for the Fagaceae project (www.Fagaceae.org) and upon cutting-edge genomics and bioinformatics platforms at Penn State University and the Clemson University Genomics Institute. Participants in the current phase of the project include Stephan Schuster, Webb Miller, Charles Addo-Quaye and Lynn Tomsho at Penn State University, along with Meg Staton (and previously Stephen Ficklin) at Clemson University.

The primary goal for the Chestnut genome project is to produce a high-quality reference sequence for the genome of *Castanea mollissima* cv Vanuxem. To assist in the identification of genes associated with resistance to *Cryphonectria parasitica*, genome sequences will also be produced for American chestnut and for other genotypes of Chinese chestnut. It is hoped that the broader impact of this project will be to demonstrate the power of genomics to address the increasing forest health and ecosystem restoration issues that is faced now and in the future.

Scientific Approach. Their plan follows a two year time line in which we will produce up to 25-fold depth of genome sequence coverage using the Roche 454 pyrosequencing technology, and at least 30-fold depth of coverage with the Illumina Solexa sequencing technology, followed by *de novo* assemblies, transcript mapping, gene identification, and gene annotations. Use of both the 454 and Illumina complementary "next generation" DNA sequencing technologies has been found to provide the best coverage, fewest gaps, and most reliable gene sequences for new genomes. The Vanuxem cultivar was chosen for the reference genome because of its key role in The American Chestnut Foundation®'s breeding program (http://www.acf.org/) and the Fagaceae Genomic Tools project. The reference genome will be assembled de novo from 454 sequence data, corrected and extended with Illumina sequence data, and pseudo-chromosomes built by integration of the physical and genetic maps for Chinese chestnut from Tom Kubisiak and Bert Abbott's programs . Candidate genes for disease resistance will be identified from genome regions spanning the blight resistance QTL. Functional analyses of the candidate genes will be conducted by the FHI's Transformation & Clonal Testing project team lead by Bill Powell and Chuck Maynard at SUNY-ESF, and Joe Nairn and Scott Merkle at the University of Georgia. To facilitate the discovery of blight resistance genes, additional genomes will be produced by "resequencing" using the Illumina technology, including Mahogany and

other varieties of Chinese chestnut, a blight-sensitive American chestnut, and possibly blight-resistant hybrids. SNPs spanning the genome will also be discovered and put into use by TACF® to accelerate breeding, with assistance of the FHI Genetic Technologies group, lead by Dana Nelson of the USDA Forest Service.

Progress, to date. Genome sequencing commenced at Penn State in January, 2010, and is presently at approximately 15X depth (12.8 Gb) using the 454 pyrosequencing platform, and 45X of sequence (37.5 Gb) from the Illumina sequencing platform. Preliminary *de novo* assemblies of the genome sequence have been conducted by the Clemson University Genomics Institute (CUGI) at 1X, 10X, and 15X depths of sequence data for assessment of quality and to determine a target sequence depth needed to ensure a high-quality genome. In the current, preliminary build (number 6), app 859 Mb has been assembled, which is quite close to the estimated genome size of 800Mb for Chinese chestnut (Kremer et al 2007). However, the number of contigs (969,409) and scaffolds (93,227) is currently far too high and lengths too small (N50 of 1905 bp and 3847 bp, respectively) for a useful reference genome. Additional sequencing of genomic DNA using the 454 technology is underway, focusing primarily on "paired-end" sequences of genomic DNA fragments of several lengths, to aid in the assembly of the sequence contigs into larger scaffolds. Research at CUGI includes sequencing the ends of the minimal tiling path set of BAC clones obtained from the chestnut physical mapping project. BAC end coverage of the genome is also expected to greatly benefit the conversion of sequence contigs into larger genome scaffolds. Finally, we will use a multiplexing approach to sequence BAC-contigs, from the physical map, which are located at the blight resistance QTL. This ensures the highest quality and accuracy of candidate gene sequences identified in the QTL.

We have also started the re-sequencing of additional chestnut genomes. Our first re-sequencing run on the Illumina platform was conducted for an American chestnut genome. The genotype selected, 'GladeMountainBig' ('GMBig') has also been used by CUGI to construct a BAC library for American chestnut. Approximately 40X depth of Illumina sequence (32.7 Gb) was produced for American chestnut. The American chestnut genome sequence data will be assembled by mapping of sequence reads onto the Chinese chestnut reference genome, as soon as the reference is available.

A publically accessible web portal for the chestnut genome project is being created at the Fagaceae Genomics website. The public web portal (http://www.fagaceae.org/FHI) will provide a searchable database for access to the genome sequences, along with an interactive browser for viewing the assembled genome, as soon as the assembly is finalized and properly annotated. A genome website has also been initiated at NCBI (the National Center for Biotechnology Information) to house the data and facilitate access by the greater research community to the final results of this project (http://www.ncbi.nlm.nih.gov/genomeprj?term=chestnut).

Gary Micsky, Penn State Extension Service, Mercer County, PA

NE-1033 participants and TACF® are valued and effective partners in his natural resources extension education programming, and they have been critical to his success in expanding their outreach to new audiences and have enhanced the quality of existing programs.

Methods:

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

Evaluation Process:

- Number of research orchards established
- Volunteers who learn to identify reproductive structures and correctly prepare female flowers for pollen exclusion and eventual controlled pollination
- Success of pollination efforts (yield = 79 F_1 cross nuts)
- Number of trained volunteers and volunteer hours

Volunteer Role:

- Tree ID, breeding, record keeping, culture and aftercare, program delivery
- Host research/demonstration plots
- Collect/supply genetic material
- Assist in TACF® and other research activities as needed

Volunteer Recruitment, Development, and Utilization. October 7, 2009. Chestnut Seed Harvest Volunteers (N=4) harvested and processed 123 2009 F_1 cross and approximately 5,000 open pollinated American chestnut seed for use in local and TACF® plantings in 2010. Volunteer Hours: >20.

November 19, 2009. Delivered presentation on history of American chestnut and conducted growers school for Northeast Ohio Forest Landowners Association (NEOFA) members (N=39) in Canfield, Ohio. Interested participants (n=25) were provided open pollinated seed and supplies to participate in grower/site evaluations designed to identify future volunteers and sites for restoration efforts. Of these, two participants became active volunteers in our NW PA chestnut program and have assisted in pollination efforts for PA-TACF® in Mercer County and inoculation of trees in Graves Orchard at Penn State's Research facility in Rock Springs, PA.

February 13, 2010. Asian Gall Wasp gall collection in support of Lynn Riske-Kinney's research project to monitor the occurrence of introduced and native predators/parasites of the Asian Gall Wasp. Volunteers: (N=6) Volunteer Hours: 18.

February 20, 2010. Delivered presentation *"American Chestnut – Part of Your Woods?"* Forest Landowners Conference, Fryburg, PA. 90 participants Topics included: history of American chestnut, restoration efforts, and volunteer recruitment.

March 24, 2010. "American Chestnut Site Selection and Aftercare Workshop" Mercer, PA, 30 participants.

March 26, 2010. "American Chestnut Site Selection and Aftercare Workshop" Tionesta, PA, 13 participants.

March 31, 2010. "American Chestnut Site Selection and Aftercare Workshop" Washington, PA, 20 participants.

April 3, 2010. Planted Washington County Chestnut Demonstration Orchard for the Southwestern Pennsylvania Woodland Owners Association (SWPWO). 14 participants – 21 volunteer hours.

May 20, 2010. "Ohio River Watershed Challenge."Restoring Native Species" American chestnut station. Students from several western PA high schools learned how the chestnut blight affected the resource and current efforts underway to restore the specie, 78 participants - 5 volunteer hours.

April 22, 2010. "American Chestnut Restoration, Science and Volunteers Making a Difference ", Notre Dame School, Hermitage, PA. Students and teachers learned about chestnut restoration efforts and local volunteer opportunities, 175 participants.

June 8, 2010. Six volunteers assisted in inoculations at Graves Orchard, 30 volunteer hours worked plus 8-hour travel time.

June 21, 2010. Mercer, PA Pollination School. Twelve participants received classroom instruction from Sara Fern Fitzsimmons, Northern Appalachian Region Science Coordinator on efforts of PA-TACF® and PSU in developing blight resistant American chestnut. Topics included: history of American chestnut and impact of *Cryphonectria parasitica*, TACF® breeding program, and controlled breeding of chestnut.

June 21, 2010. Sixteen volunteers received hands-on activities including a trip to Haun Orchard to assist in bagging and record keeping required for support of Joe James and Paul Sisco's *Phytophthora* resistance study.

Pollination for screening Phytophthora-resistant lines of advanced backcross lines of Graves source of resistance.

August 11, 2010. Bur Collection and Shipment to Scott Merkle, University of Georgia Volunteers: (N=2), Volunteer Hours: 5.

Securing new lines for tissue culture of American chestnut--Over 212 hours of service involving 40 volunteers to date (September 3, 2010). Note: This does not include volunteer hours required for orchard or small plot maintenance.

Ron Sederoff, North Carolina State University

The Fagaceae Genome Project. Members of this project include: Bert Abbott (Clemson University); Sandy Anagnostakis (Connecticut Ag. Exp. Station); Kathleen Baier (SUNY-ESF); Ali Barakat (Clemson University); John Carlson (Penn State University); Stephen Ficklin (Clemson University); Tom Kubisiak (USFS, Southern Institute of Forest Genetics); Fred Hebard (TACF®); Bill Powell (SUNY-ESF); Ron Sederoff (North Carolina State University); Paul Sisco (TACF®), Meg Staton (Clemson University); Chris Smith (North Carolina State University); Dahlia Nielsen (North Carolina State University); Jeff Tomkins (Clemson University); and, Nick Wheeler (North Carolina State University). In March, 2004, a small group met at the NC Biotechnology Center to develop a genomics project for chestnut. The results, two years later, in 2006, was the project, "Genomic Tool Development for the Fagaceae", NSF Plant Genome Project. The goals were:

- Develop chestnut as a genomic model for the Fagaceae
  - (oaks, chestnuts and beeches). Initial goal was to work solely on chestnut; however, NSF indicated that for grant funding, other Fagaceae members needed to be included in the project.
- Develop the genetic and physical mapping resources needed to identify resistance genes by genome wide or targeted sequencing.

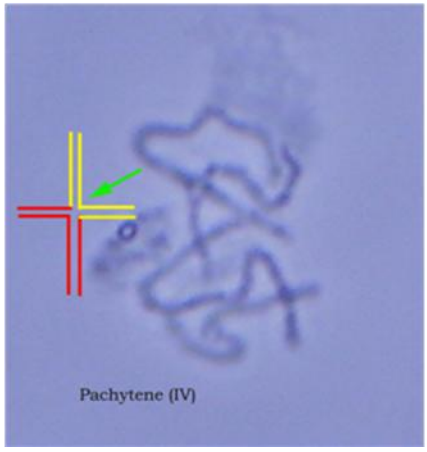
- Species to be studied:
  - Chinese chestnut (*Castanea mollissima*)
  - American chestnut (*Castanea dentata*)
  - Northern red oak (*Quercus rubra*)
  - White oak (*Quercus alba*)
  - American beech (*Fagus grandifolia*)

The major objectives were:

- 454 EST sequencing of cDNA libraries from four species of the Fagaceae. Focus on Chinese chestnut.
- Development of a large set of SNP and SSR genetic markers.
- Establishment of new mapping populations in Chinese and American chestnut.
- Genetic mapping in Chinese and American chestnut and derived hybrids.
- Verify QTL mapping for resistance to chestnut blight.
- Creation and analysis of a BAC library of Chinese chestnut.
- Construction of a physical map for Chinese chestnut.
- Integration of the genetic and physical maps.
- Create a platform for genome sequencing and map based cloning of three resistance QTLs/genes.
- Establish a web site to make all information available.

Using 454 cDNA sequencing on five species, the following has been accomplished:

- ~454 million bases read, on 10 libraries
- 120,000 total contigs
- 21,000 contigs are >700bp
- 10,562 genes were defined in all of the species
- ~800 SSRs
- ~12,562 SNPs (~1/800 bp)
- Many potential markers within and between species.
- About 15% of the genes are not found in *Arabidopsis*, but they are homologous with to sequences in Poplar. Bert Abbot's strategy was to focus on expressed genes. Sequencing and polymorphisms were all based on expressed genes. That meant that when genes were mapped, back clones could also be identified. That procedure was followed.
- There are indications of chromosomal rearrangements between *C. mollissima* and *C. dentata*. In a comparison of European and American chestnut/Chinese chestnut hybrid maps, it was found linkage group B missing. High segregation distortion in both the F_2 and BC_1 maps (20 to 31%). Ron Phillips proposed that rearrangements have occurred between the two species.



Nurul Islam-Faridi (TAMU).
Visible quadrivalent in Chinese/American hybrid meiosis (pachytene)

Clear evidence of rearrangement.
Possibly multiple rearrangements of different kinds.

This is important if you are going after genes in chromosomes by map-based cloning. Good maps are needed and problems such as this need to be resolved.

Mapping. New mapping populations were needed and created. EST sequencing provided large numbers of SNPs and SSRs. High throughput SNP genotyping used the *Illumina* Golden Gate Assay (a multiple-oligo-ligation-based PCR assay) that detects allele specific sites for 1536 markers simultaneously. Sederoff showed a map of Chinese chestnut with some specific markers in red. If a Chinese chestnut parent that is part of the hybrid breeding program is used, markers can be tagged so that it can be determined what proportion of the genome is Chinese in a specific individual and what regions are American. In that way, one can not only associate genes with resistance and susceptibility, but other regions of the genome that have characteristics that can be tagged to select for or against a certain trait.

Physical mapping. The genome size of chestnut is about 800 Mbp. The physical map was done on a newly discovered genotype called Vanuxem Chinese chestnut. Two BAC libraries were constructed (EcoRI and HindIII—126,455 BACs Coverage = 17X) and analyzed by fingerprinting and contig building. Then hybridizations were used to place ESTs on BACs. Mapping SSRs and SNPs places BAC contigs on linkage maps. There are now 1,377 contigs with 12,919 singletons. The coverage was 1.2X.

Web page. The webmaster is Meg Staton. The website is: www.fagaceae.org. The website contains information on: physical mapping; genetic mapping; sequences; marker development; and, all reports and presentations made by project members.

Sederoff concluded by stating that the group was right to focus on ESTs, 454-sequencing, SNPs, SSRs and BAC cloning. The result was a set of genomic resources for:

- Full genome sequencing
- Map-based cloning of resistance genes
- Genome-wide association studies
- Marker-aided introgression
- Comparative genomics of the Fagaceae
- Functional genomics of adaptation

Dana Nelson, USFS-Southern Institute of Forest Genetics

Advancing forest health through biotechnology: Forest Health Initiative (FHI). Nelson explained the goal to integrate the newest technologies with existing materials and know-how to accelerate development of resistant trees for species restoration. At the same time, it is hoped that the following can be engaged: stakeholders on social and developmental issues and regulators on requirements for genetically-modified forest trees. In the FHI, there are three working groups:

- Science
    - Genomics (genes and markers)
    - Germplasm (gene mapping and marker-assisted breeding)
    - Clonal/transgenics (cloning and genetic modification)
- Social and Environment
    - Restoring a native forest species
    - Biotech trees in the forest
- Regulatory and Policy
    - Transgenic trees (permits through deregulation)
    - Rules for forest trees, restoration
        - Long-lived forest and nut trees
        - Promote transgene spread to native gene pool

The Science Group builds off: a long history or chestnut genetics, pathology and breeding; and, the recent history of NSF Fagaceae Genomics Project. The institutions involved include: North Carolina State University; Penn State University; Clemson University; and, the USFS. The coPIs for the Science Group are John Carlson (Penn State University); Bill Powell (SUNY-ESF); Scott Merkle (University of Georgia); and, Dana Nelson (USFS). Members of the science advisory committee include: John Davis (University of Florida, chair); Steve Strauss (Oregon State University); Rod Sederoff (North Carolina State University); Toby Bradshaw (University of Washington); and, Nick Wheeler (consultant).

The research goals are of each group are:

- Genomics group
    - Provide reference genome sequence of Chinese chestnut
    - Find sequence difference between Chinese and American chestnut
    - With the germplasm group, identify and sequence all genes in the blight resistance QTLs
- Germplasm group
    - Fine-map disease resistance QTLs
    - Develop marker-assisted breeding tools
    - With the transgenic group, develop early screen for blight resistance and evaluate resistance with clonal replicates
- Clonal/Transgenic group
    - Test somatic embryogenesis technology in a wide array of materials (American chestnut to advanced hybrids)
    - Develop transgenic technology for American chestnut
    - Test candidate resistance genes, especially from Chinese chestnut
    - Test early flowering genes for accelerated breeding

Bill Powell, SUNY-ESF

American chestnut research and restoration project at SUNY-ESF. Powell provided an overview of the work being conducted at SUNY-ESF. One question is, "will transformed trees reproduce?" Trees derived from tissue culture have now gone through the entire process, from embryos to mature, nut producing trees. After 7-8 years, these tissue culture-derived trees have produced their first crop of nuts. In 2009, these same trees produced female flowers but they all aborted.

Powell showed transformation events that were brought up to shoot stage and beyond. Once the shoot stage is obtained, countless trees can be produced. Definition of an "event: When a transformation is done, the gene construct has moved into the plant genome into a certain location. In a transformation experiment, there might be 0-5 events. Numerous individuals from each event can then be produced by clonal propagation. This is important, because when the gene goes into the genome, the surrounding area can affect how that is expressed. Different events can have different levels of expression. Powell strives for 10-20 events to look at all the different expression patterns.

Vector(s)	Promoter and candidate genes	Total transgenic events (as SEs)	Events as TC shoots	Events in the field
pESF-KBO/pTACF3	Vascular (VspB) promoter Oxalate oxidase (OxO)	19	13	3 (~208 trees)
pTACF7	VspB-OxO + ACS2-ESF39 AMP	4	4	2 (~61 trees)
p35S-OxO	CaMV 35S-OxO	16	16	
pESF-KBLOE	ACS9-Laccase + VspB-OxO + ACS2-ESF39 AMP	10	7	
P35-CNO	CaMV 35S-chitinase/NIa proteinase/OxO	11	11	
pESF-KBL	ACS9-Laccase	3	2	
pWVK147	Empty control vector	2	2	

So far, his field plantings have been involved with the above five events. With the new ones for next spring, there should be a minimum of 50 events (5 trees/event = 250 trees) in 2011.
Powell expounded on oxalate oxidase, an enzyme found in wheat that breaks down oxalic acid into hydrogen peroxide and carbon dioxide. This is important because *C. parasitica* produces a lot of oxalic acid at the canker margin, a compound used by the fungus to lower the pH. The idea is that oxalate oxidase will detoxify that product of the fungus. The 2009 plantings in central New York have some of the first good expressers of the wheat oxalate oxidase gene.

Powell tries to get the public involved with transgenic trees. In 2010, there were planting in eastern (Lasdon Botanical Garden, Somers, NY) and western New York (outside of Buffalo). Plans in 2011 include northern New York (Watertown) and a demonstration in North Carolina.

Transformants made so far, from first generation genes, are all from non-chestnut sources. They are now cloning second generation genes from Chinese chestnut.

Cloned second generation candidate genes (CG) from Chinese chestnut

Chinese CG (putative ID)	**cDNA clone**	**Binary vector(s)**	**Events**	**Shoots**
Laccase (diphenol oxidase)	S	pESF-KBLOE pESF-KBLO pESF-KBL	10	7
β-1,3 glucanase		pFHI-B13Gluc	3	2
CBS domain protein (unknown function)		pFHI-CBS1		
UDP glucosyltransferase				
Thaumatin-like protein				
Acid phosphatase				
Shikimate 5-dehydrogenase				
Proline-rich protein (similar to PRP3 in *C. sativa*)				

They have cloned several genes from Kathleen Baier's full-length cDNA library, identified through suppressive, subtractive hybridization libraries as well as in data from Ali Barakat and the Genomics project. The thaumatin-like proteins is located in the middle of resistance locus LG G (noted by Tom Kubisiak). It is a putative allergenic compound.

Powell has developed a new collaboration from the Forest Health Initiative. Tim Tschaplinski and others have run some of the SUNY-ESF samples in the mass spectrophotometer at the Oak Ridge National Laboratory. Kathleen Baier was conducting a time course inoculation (0, 3 hr, 24 hr, 72 hr and 7 days) in Chinese and American chestnut. Some of the ground tissue was sent to Tschaplinski. The analyses are not complete, but Tschaplinski noted that, "American chestnut has a lot more of what any microbe wants and Chinese chestnut has a lot more of what microbes do not want." These data should help in choosing next candidate genes for up- and down-regulation.

Another goal of the FHI is to transform an early flowering gene into chestnut, to shorten generation time. This has been done in plum in Ralph Scorza's lab. The Powell lab obtained two contructs from Steve Strauss (Oregon State University) that have the Poplar FT1 gene. One construct is under the control of a heat shock promoter and the other is under the control of the 35S promoter, which is expressed all the time. The idea is to get flowering in the greenhouse early on to do a lot of breeding and then later breed out the transgenic gene to have untransformed chestnut. They are almost to the point where they can confirm that they have some events.

Kathleen Baier, SUNY-ESF

Differential expression of a putative laccase gene. Baier is interested in how the expression of the laccase gene differs between American and Chinese chestnut. In many plants (Arabidopsis, Pine, Poplar), laccase is a member of a multigene family. It functions in phenolic metabolism, so it may play a role in secondary metabolites and lignin production. The nature of many secondary metabolites is antimicrobial along with lignin production (part of the structural components of trees) to set up barriers against microbes. Both components may be involved in plant defense response against pathogens. This may be why laccase shows a differential expression in American and Chinese chestnut.

When she was working on her thesis, she looked at gene expression in American and Chinese chestnut trees. One of the things she did was a Northern blot looking for expression of a putative laccase. She looked at six American and six Chinese chestnut seedlings (7 days post inoculation; 7 days post wounding; and, non-inoculated healthy stem tissue). The probe in this case was designed for a short sequence of Chinese chestnut putative laccase (at the time that was the only sequence that they had). The assumption was that the two species would have similar enough sequence that they could detect expression in American chestnut. She did not detect any expression in the six American chestnuts using this probe. Some of the questions that arose from this finding were: "Is this gene even in American chestnut?" and "Was the sequence so different that they could not detect it with a probe that was specific for Chinese chestnut?" and "Was there a gene in Chinese chestnut with a similar sequence that was being expressed so differently that it could be detected by this method?" After seeing this result, Baier wanted to try to isolate the full-length Chinese chestnut laccase and obtain the sequence. She was able to isolate a full-length Chinese chestnut clone and sequence it. She used some primers that would give her the full open reading frame in Chinese chestnut and locate it in American chestnut cDNA library. This cDNA library was made from a pool of tissues (stem, leaf and bud tissue), and it was fairly easy to use PCR to pick up a full-length cDNA clone in that American chestnut library. When she compared the two cDNA sequences, they were fairly similar, but the predicted amino acid sequence was 100% identical. These data are shown in the following table.

BLASTx of Chinese chestnut laccase open reading frame amino acid sequence similarity

Description	Max identity	E value
Putative laccase/diphenol oxidase (*C. mollissima*)	100%	0.0
Putative laccase/diphenol oxidase (*C. dentata*)	100%	0.0
Laccase, putative (*Ricinus communis*)	68%	0.0
Multicopper oxidase (*Populus trichocarpa*)	64%	0.0
Laccase (*Acer pseudoplatanus*)	64%	0.0
Laccase (*Toxicodendron vernicifluum*)	60%	0.0
Laccase (*Litchi chinensis*)	58%	0.0
Putative laccase (*Rosa* hybrid cultivar)	53%	5e-175

She wanted to look at a more quantitative expression level so she used RT-qPCR and when doing that, she wanted to have some gene specific primers (primers were designed between positions 1281-1361). She did not want to pick up another laccase in chestnut. She designed primers that would bind identical regions in both American and Chinese chestnut. Using inoculated American and Chinese

chestnut trees (0, 3 hr, 24 hr and 168 hr), Chinese chestnut expressed laccase at a significantly higher level than American chestnut. At the 7 days post inoculation (168 hr), laccase expression in Chinese chestnut was six times higher than American chestnut from stem tissue. She then looked at other tissues (leaf and seeds). There was no significant difference in leaf tissue, but there was significant difference in seeds (Chinese chestnut had about 150-fold higher level of laccase expression than did American chestnut). She looked at laccase expression in newly expanded and mature Chinese chestnut leaves and there was significantly higher expression in the mature leaves.

In summary, laccase may be important in the disease defense response because of what it does functionally in phenolic metabolism and also because it is much more highly expressed in Chinese than in American chestnut.

Andy Newhouse, SUNY-ESF

Early screening assay; excised leaf inoculation. Newhouse is looking at something that can be done earlier in the process of developing transformed trees to identify resistance. Small, germinating seedlings are too small for a stem assay. An early assay should be:

- Simple
- Effective on younger plants
- Non-destructive to plants
- Meaningful—correlated with established inoculation protocols
- Quantitative (able to detect partial resistance)

His goal is not to predict perfectly how a tree will respond but to get a rough idea of events that they may be interested in pursuing versus events that are more susceptible.

Detached leaf assay is not a new idea. It has been used in other systems. Leaves have been used to screen trees for Sudden oak death. The SOD pathogen is *Phytophthora ramorum* and the hosts are *Lithocarpus* and *Quercus*. Various methods have been used in SOD at UC Berkeley:

- Agar plugs pinned to attached leaves
- Agar plugs on excised leaves
- Petiole-plug on excised leaves

The process used by Newhouse is as follows:

- A #1 cork borer is used to excise inoculum from the margin of 4-7 day-old PDA cultures of *C. parasitica*
- Collect leaves, label them and define 5mm sections on leaf margin where inoculations will be made (2 inoculations per leaf are made)
- Use scalpel to make a slit along the 5mm marks (cut to half the depth of the midvein)
- Place agar plug on wound
- Incubate in sealed container for one week
- Measure length of necrosis along the abaxial midvein (he has subcultured from necrotic area and successfully isolated the *C. parasitica* isolate used to initiate the lesion)

Variations in his experimental factors tested to date include:

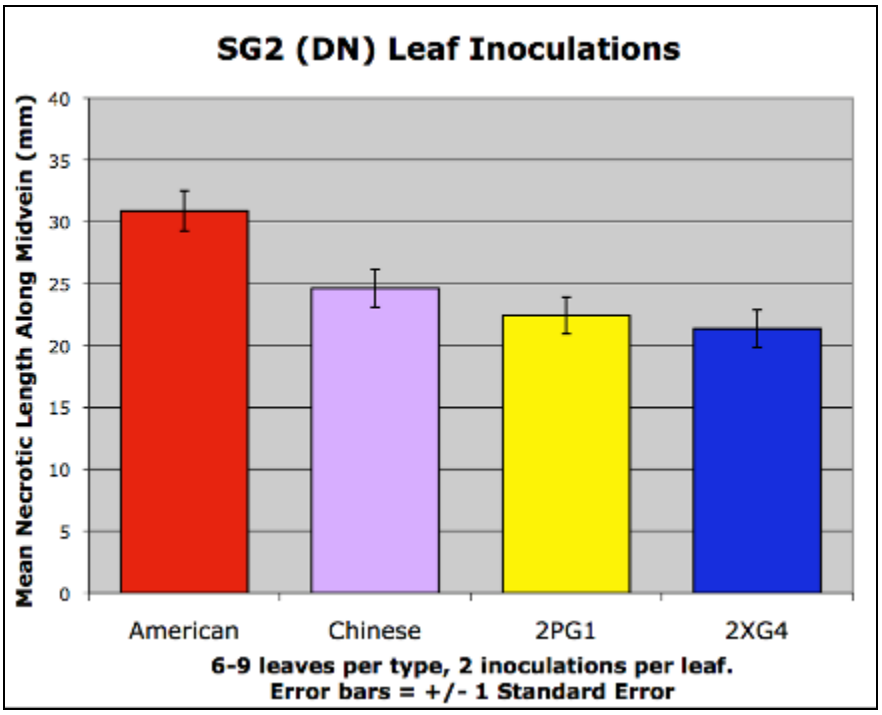
- Rubber gasket-sealed pans are better than Saran wrap or plexi-covered chambers.
- Dark, slightly damp conditions allow for good fungal growth; these conditions reveal more variation between leaf types.
- He has used different wound types. To date, he has found that the pin/scratch wound is less effective than the scalpel slice and liquid is less convenient and effective than agar plugs.

- The distal end of the leaf is more susceptible that the petiole end

Results:

- There is a good deal of variation, so averages are key.
- Chinese chestnut leaves consistently show less necrosis than American chestnut leaves.
- Leaf necrosis correlates with pathogen virulence.

Newhouse tested necrosis on transgenic trees using *C. parasitica* isolate SG2-3,. His data indicate that necrosis, resulting from leaf inoculations, on American leaves is significantly different than Chinese and AN-2XG4 and KS-2PG1 as shown below. The AN-2XG4 (called Darling 4) contains the oxalate oxidase gene and KS-2PG1 (called Hinchee 1) contains the oxalate oxidase gene plus the antimicrobial peptide gene (ESF39). Both of these also contain selectable and scorable marker genes NPT2, BAR, and GFP:



Katie D'Amico, SUNY-ESF

Evaluating ectomycorrhizal colonization of transgenic, hybrid and wild type *C. dentata*.

Mycorrhizae are fungi that colonize the roots of most plants. It is a mutualistic relationship between the fungus and the plant. Fungal hyphae extend the root system of a plant and the fungus is able to access nutrients in the soil that would be otherwise unavailable to the plant, such a phosphorous. In return, the plant provides carbohydrates for the fungus. Nutrient exchange occurs where the fungal mantle interacts with the plant epidermal layer. All Fagaceae species are ectomycorrhizal.

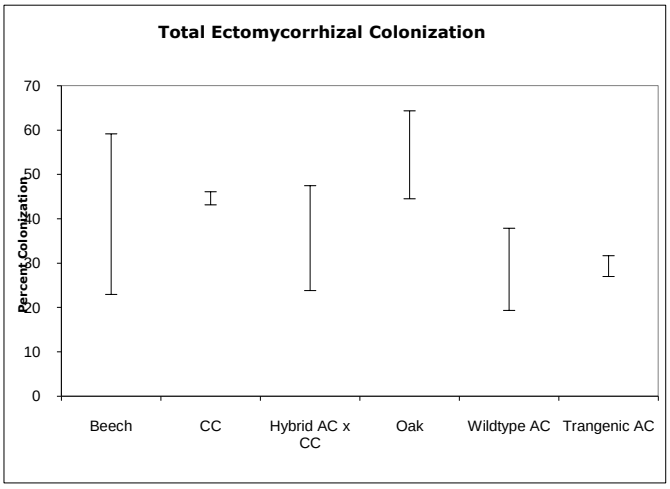
There may be some physiological changes in the transgenic trees as a result of gene modifications, so they pay particular attention to make sure that there are not any detrimental effects on the mycorrhizal relationship. D'Amico's project involves ectomycorrhizal colonization in one particular transgenic line with wheat oxalate oxidase (OxO).

D'Amico looked at other studies using transgenic plants (poplar, silver birch and elm) and she found no change in mycorrhizal colonization between normal and transgenic plants. D'Amico is doing

a greenhouse study, so she is growing seedlings in a greenhouse and she will then assess mycorrhizal colonization. She is using six tree types:

- Transgenic American chestnut ('Darling')
- Wild type American chestnut ('Zoar')
- Chinese chestnut ('Hong Kong')
- American-Chinese hybrid (BC_1)
- Northern Red oak (*Quercus rubra*)
- American beech (*Fagus grandifolia*)

Trees grown in a greenhouse do not typically form mycorrhizal associations. In order to get colonization in the greenhouse, she mixed field soils with potting mix and sand. She used soils from two sites (lower shelterwood and uppershelterwood). She planted six trees of each type into each soil type for a total of 72 trees. To assess mycorrhizal colonization, she grew the seedlings in the greenhouse for 1 year. She then removed the plant from the pot, rinsed the root system over a sieve and collected root pieces (12 pieces of 4-6 cm long roots). She looked at the roots under a dissecting microscope and classified root tips as colonized or uncolonized. D'Amico grouped colonized tips by morphotype and stored them for molecular analysis (RFLP). Her data of total colonization is seen in the following graph.



D'Amico's conclusions were:

- Field soils serves as a good inoculums for ectomycorrhizae.
- She will determine species by RFLP and compare species percent colonization.
- She will use qRT-PCR to examine transgene expression in roots, stems and leaves.
- She will compare results to those of the field component of this study.

Tom Kubisiak, USDA, Southern Institute of Forest Genetics

Project Milestones:

Complete the genetic map of Chinese chestnut. Last year he reported our progress on the construction of a genetic linkage map for Chinese chestnut (*Castanea mollissima*). Since that time last year, all data collection has been completed (May 2010). A final integrated consensus linkage map has now been constructed. Segregation data for a total of 1401 markers, 330 simple sequence repeats (SSRs) and 1071 single nucleotide polymorphisms (SNPs), were available for map construction use two related intra-specific Chinese chestnut crosses, 'Vanuxem' x 'Nanking' (n=157) and 'Mahogany' x 'Nanking' (n=179). Of the 1401 markers, 1156 were confidently ordered using conservative thresholds. A consensus map was constructed that consists of 12 linkage groups spanning 742.4 cM with an average locus spacing of 0.7 cM. Putative gene function was assigned to the mapped ESTs using BLASTx and the Swissprot protein database for plants. Approximately 37% of all mapped ESTs were confidently assigned a putative gene function. To these, European Commission enzyme codes (EC codes), protein motifs based on InterProScan, and Gene Ontology (GO) terms were assigned if possible. Third level GO assignments for each of the three functional categories; molecular function, biological process, and cellular compartment suggest that the set of ESTs to which putative gene function could be assigned represent a large number of sub-categories reflecting a widely diverse set of gene transcripts. BLASTn analysis was used to compare the genetic map for Chinese chestnut to the physical maps (whole genome sequences) of two model tree species, peach (*Prunus persica*) and black cottonwood (*Populus trichocarpa*). Mapped ESTs that had only one significant hit to the peach genome and those that had either one or two significant hits to the cottonwood genome were considered for comparative analysis. Visual and statistical analysis suggested that there were a number of significant segmental homologs between chestnut and peach as well as between chestnut and cottonwood. Regions of extensive macro-scale synteny and collinearity exist between the chestnut genome and that of both peach and poplar. As dense genetic maps and whole genome sequences become available for more tree species, further detailed examination of genome organization and evolution may lead to important insights into the effects of genome structure on various phenotypes. A manuscript is currently being written that describes this work.

Construct a genetic map for American chestnut. Significant segregation distortion has been noted in several different hybrid crosses between American chestnut and Chinese chestnut. In inter-specific crosses, segregation distortion is thought to be caused primarily by chromosomal rearrangements between the species that result in abnormal pairing and disjunction during meiosis in the F_1. Recently, Islam-Faridi et al. (Acta Hort. (ISHS) 844:207-210, 2009) provided cytogenetic evidence of a translocation, as a quadrivalent in pollen mother cells of an F_1 hybrid between Chinese chestnut and American chestnut. If rearrangements are associated with chromosomes carrying resistance factors, introgression of resistance in backcross breeding may be hindered. To further address the nature and extent of potential genomic rearrangements between Chinese chestnut and American chestnut, a genetic linkage for American chestnut is being constructed. During the course of this past year, a preliminary genetic linkage map was constructed for American chestnut (*Castanea dentata*). Given that very few Chinese chestnut SNPs were found to be polymorphic in American chestnut, a new SNP platform was developed. A SNP array consisting of 768 polymorphisms was developed from American chestnut ESTs that had high BLAST identity to mapped Chinese chestnut ESTs. This approach was taken to maximize the correspondence between the Chinese chestnut and American chestnut genetic maps. The 768 SNP array was used to genotype an intra-specific American

chestnut cross developed by Fred Hebard (GMBig x Horn; n=166). Of the 768 SNPs, 259 were segregating in the GMBig x Horn cross. The data were scored and a preliminary genetic linkage map for American chestnut was constructed. The map consists of 12 primary linkage groups and preliminary comparisons to the map for Chinese chestnut suggest that there may be some structural differences between the two genomes. However, although there appears to be some evidence for potential rearrangements such as putative inversions, there was no obvious evidence for any reciprocal translocation events in one species relative to the other. We are in the process of collecting additional SSR data to expand the map for American chestnut and will be doing further analyses to investigate the nature and extent of potential rearrangements and their correlation with regions in the hybrid F_2 genome that are experiencing significant segregation distortion.

Delineate the region of the Chinese chestnut genome that is responsible for resistance to the blight pathogen. Given the new genetic resources, more specifically the large number of codominant genetic markers that have become available over the past several years from the NSF Fagaceae project, there existed the opportunity to re-examine the existing F_2 mapping population originally used to define QTL for resistance to *C. parasitica* (Kubisiak et al., Phytopathology 87:751-759, 1997). The goals of this study were to use the newly developed genetic marker framework to: 1) more precisely define the location of blight resistance QTL; 2) better define the allelic effect of resistance QTL (dominant, partially dominant, codominant, recessive); 3) correlate marker-defined QTL regions with the genetic map for Chinese chestnut; and, 4) identify potential candidate disease resistance genes. Over the course of this past year, segregation data was collected for the 1997 inter-specific Chinese chestnut x American chestnut F_2 population (n=83) using the 1536 Chinese chestnut SNP array. Approximately one-third (447) of these markers were heterozygous in at least one of the F_1 parents and hence useful for mapping. Of these, 70% were heterozygous in both parents and hence afforded the opportunity to better define the allelic effects of QTL. Genetic maps were constructed for each parent. The maps consisted of 12 linkage groups and no unlinked markers were observed, suggesting complete coverage of the hybrid genome of each parent. As had been noted previously, significant segregation distortion was again observed (~24% of all SNP markers). Some minor levels of segregation distortion were noted on most linkage groups, except linkage group D, with excessive distortion noted on E, K, and L. QTL analyses were performed using various approaches such as non-parametric analysis, interval mapping, and Marker-QTL-Marker analysis. Analyses were performed separately for each F1 parent using a variety of canker size metrics (computed from length and width of cankers). These included data for canker sizes incited by two different isolates (Ep155 and SG2-3) over two time periods. Based on the results, there appears to be evidence for at least 4 significant QTL, one on LG B, one on LG E, one on LG F, and one on LG G. At all QTL, resistance alleles were inherited from Chinese chestnut. The effect of QTL alleles on LG B appear to be additive and “effective" against both isolates Ep155 and SG2-3. QTL alleles on LG E appear to have a dominant mode of action and are also "effective" against both isolates Ep155 and SG2-3. QTL alleles on LG F appear to have an additive effect but this QTL only seems to be "effective" against isolate SG2-3. Conversely, the QTL alleles on LG G also have an additive effect but appear to only be "effective" against isolate Ep155. The QTL on B, F, and G were consistently detected in both F_1 parents, whereas the QTL on E was only detected in R4T52. The QTL on LG E was in a region experiencing excessive segregation distortion so this QTL still requires further evaluation. Overall, these data shed new light on the identification, mode of action, and “effectiveness” of resistance QTL. The detection of QTL that may be isolate-specific was unexpected. Certainly, these results require further evaluation and validation using other segregating populations. Additional QTL

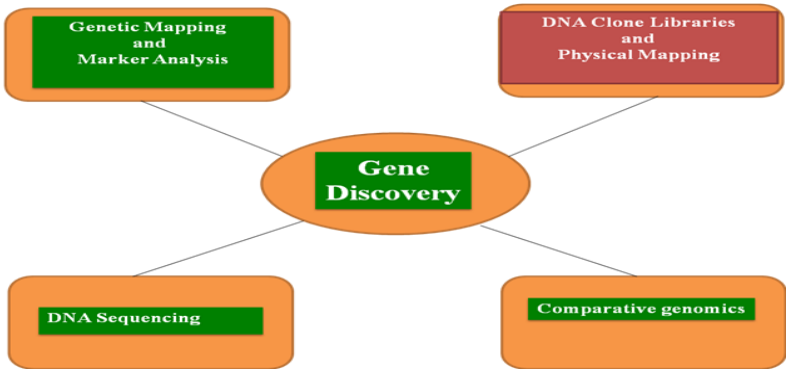
mapping is planned under the Forest Health Initiative (FHI) which will consist of more advanced lines (BC_3s and BC_3F_2s) with significantly larger population sizes (n=1000s).

In an attempt to identify potential candidate disease resistance genes, marker sequences bracketing QTL were BLAST'd against the peach genome. Interestingly, these results suggest that at least three of the four QTL (B, F, and G) for resistance to the blight fungus in chestnut may potentially be syntenic to QTL in peach for resistance to powdery mildew. This was very exciting and suggested that the sequenced peach genome might be immediately useful for identifying candidate resistance genes that could be tested in chestnut via transformation. Using this approach, a list of candidate genes was compiled. This list was then cross-referenced with a list of candidate genes identified via suppression subtractive hybridization SSH (Powell) and/or transcriptomic data (Barakat). A "short-list" of potential candidates for testing was compiled for the FHI project. Two promising candidates are a UDP glucosyltransferase and a thaumatin-like protein.

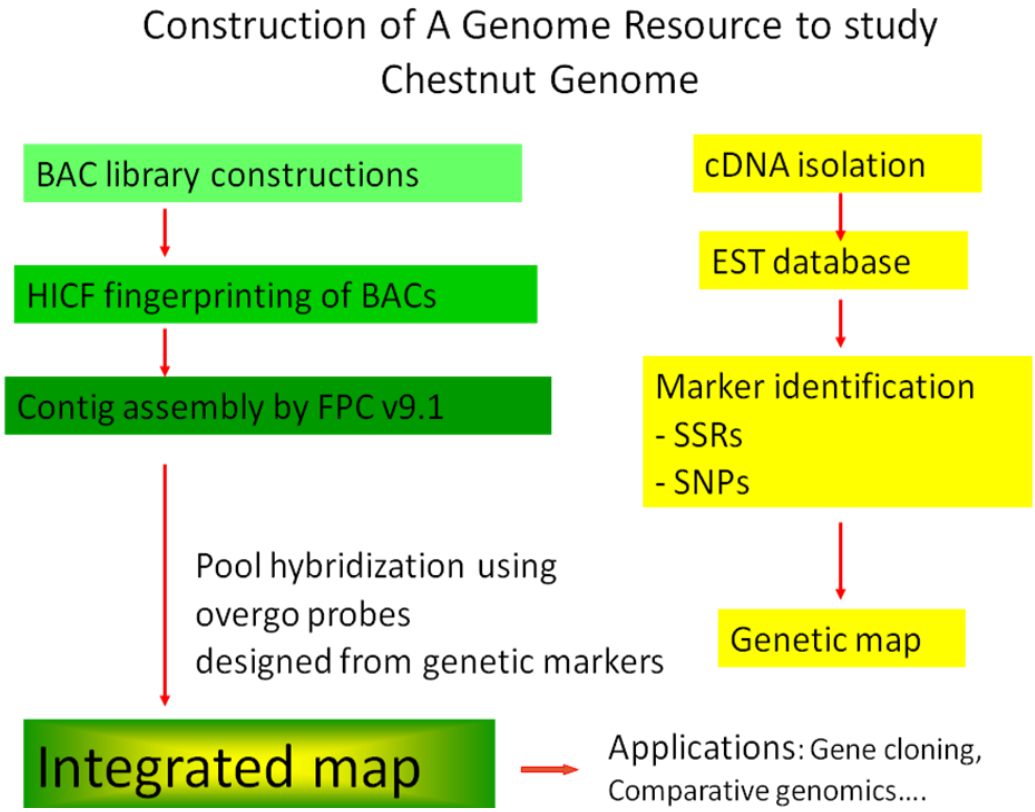
QTL analysis of *Phytophthora* resistance in a BC_1 population. From 2004-2008, a large number of chestnut and chinkapin families, including some hybrid families, were evaluated for *Phytophthora cinnamomi* resistance by Steve Jeffers (Clemson University) and Joe James (Chestnut Return Farm). In these experiments, trees were subjected to a stringent screening procedure where they were artificially exposed to 2 isolates of *P. cinnamomi* while being grown in large containers (cattle troughs filled with sterile Fafard 3B soilless mix). For one inter-specific BC_1 family (KYAdair1 x GL158; n=48) 1:1 segregation of phenotype (27 alive versus 4 struggling/17 dead) was observed. Thus, it appeared as if resistance to *P. cinnamomi* in this genetic cross might be controlled by only a single genetic factor. Tissue had been collected from these individual's prior to their demise and hence were available for genetic analysis using DNA markers. Segregation data was collected for this population using the 768 American chestnut SNP array. A total of 231 markers were segregating and used to construct a combined genetic linkage map for the parents. QTL analysis was performed using interval mapping where phenotype was scored as; 1=alive, 2=struggling, 3=dead . A single major effect QTL (max LOD 6) was detected on linkage group E that explained greater than 30% of the variation. Thus, at least in this particular family, there appears to be one single major effect locus that explains a significant amount of the variation observed. Further validation of these results using additional crosses with more extensive progeny sets is recommended. Provided that resistance to *P. cinnamomi* is controlled by only a single genetic factor in other Asian sources of blight resistance, and that resistance to *P. cinnamomi* is not being inadvertently selected against, some small proportion of advanced generation backcross lines (25% of BC1 trees, 12.5% of BC2 trees, 6.25% of BC3 trees,….) should harbor resistance to both *C. parasitica* and *P. cinnamomi*.

Albert Abbott, Clemson University

Comparative genomics and gene discovery in chestnut. Abbott reintroduced the Fagaceae team. He then talked about gene discovery, which is central to any genomics effort. Over the history of genetics and genomics, over many species, the following diagram describes what most projects have come to do in terms of genomics efforts. Very traditional mapping approaches and marker analysis, together with the more structural sequence-based physical mapping and clone libraries and DNA sequencing, lead us to new comparative genomics, where a large amount information from a lot of different species can be accessed.



Physical mapping. Below is the general strategy to get to the point where you have a physical map resource that can be used in the identification of genes, gene regions, etc.



Many genome projects follow the above strategy. There are large insert library constructions; those are the substrates used to build large physical maps to get contiguous pieces of DNA. The physical mapping construction has been carried out by various strategies related to fingerprinting to find out the overlaps. This builds up large contiguous pieces of DNA, called contigs; this is computer driven. Once the physical map is generated, the large pieces of DNA still have to be identified. This is where all the other kinds of information are integrated that allow investigators the ability to find regions of the genome which have the genes of interest. On the other side, people spend a lot of time characterizing the sequences of ESTs expressed in different tissues at different times and if you take that data and genetic marker map data and with these types of data points, you can actually use hybridization strategies and you can lay this type of data across a physical map and locate the genes and markers on the physical map. In the end, you end up with a genetic map and a physical map that is integrated with all the genetic information. One can go along and look for regions of the physical map that contain genetic markers. That is what gets you to the genes that you are interested in. The markers mark the character. You know if you get an interval that is between two markers where a character is and you find that physical location on your physical map, you have a piece of the DNA that contains the genes. That is the strategy that was done at Clemson.

The start of physical mapping is large genome insert libraries. There are four libraries and three were used for actual physical map construction from Chinese chestnut DNA and one library was from American chestnut, as shown below.

Chestnut BAC libraries

	Library			
	CMCMBb[a]	CMCMBd[a]	CM_MBc[a]	CDC_Ba[b]
Restriction digest	*Hin*dIII	*Eco*RI	*Hin*dIII	*Hin*dIII
Avg. insert size	123 kb	115 kb	90 kb	140 kb
# of clones	73,728	92,160	110,592	73,728
Genome equivalents	11X	10X	12X	12X
Filters	4	5	6	4

[a]Libraries were made from Chinese chestnut, *C. mollissima*, cultivar Vanuxem.
[b]Library made from American chestnut, *C. dentata*.

The Chinese chestnut libraries were subjected to HICF fingerprinting. The physical map has gone through a lot of work trying to get a physical map down to a series of large contigs that span significant portions of the chestnut genome. At the current point, they have 1,377 contigs of physical map DNA. On that are anchored 1,026 genetic markers. They have integrated those physical pieces of DNA with actual genetic markers. Marker data for genetic mapped markers is shown in the table below.

Hybridization with mapped genetic markers anchored physical contigs to all 12 linkage groups of the Chinese chestnut genome.

Linkage Group	# mapped genetic markers	# mapped contigs	Total physical span of mapped contigs (Mb)
A	68	44	70.3
B	49	22	39.0
C	43	23	44.6
D	51	27	51.3
E	57	33	55.9
F	33	18	34.0
G	30	23	36.9
H	50	22	39.1
I	42	29	42.0
J	40	36	47.7
K	37	25	37.9
L	46	33	46.5
Total	547	335	545.2_a

[a]The physical span is near 70% of the total genome size (800 Mb).

DNA sequencing. On one side, the physical structure is being built. The other side is other types of data feeding into this. In the NSF project, besides the physical map, there are a lot of EST transcriptome studies, based on EST sequencing of lots of different tissues under different conditions (infected vs noninfected). The transcriptome work has been going on in parallel. A whole series of libraries have been prepared. The Chinese chestnut transcriptome is very deep. The transcriptome strategy is detailed below.

Goals: identify candidate genes involved in defense against the chestnut blight fungus

Approach:

- Identify *in silico* genes induced in canker vs healthy stems from American and Chinese chestnut approach using DESseq method
- Compare *in silico*, the expression of orthologous genes from Chinese and American chestnut and identify genes induces in each species or in both of them
- Identify gene and gene networks involved in chestnut defense against the fungus
- Confirm the expression of candidate genes using real time PCR
- Map candidate genes on the physical map and identify genes located in QTL regions

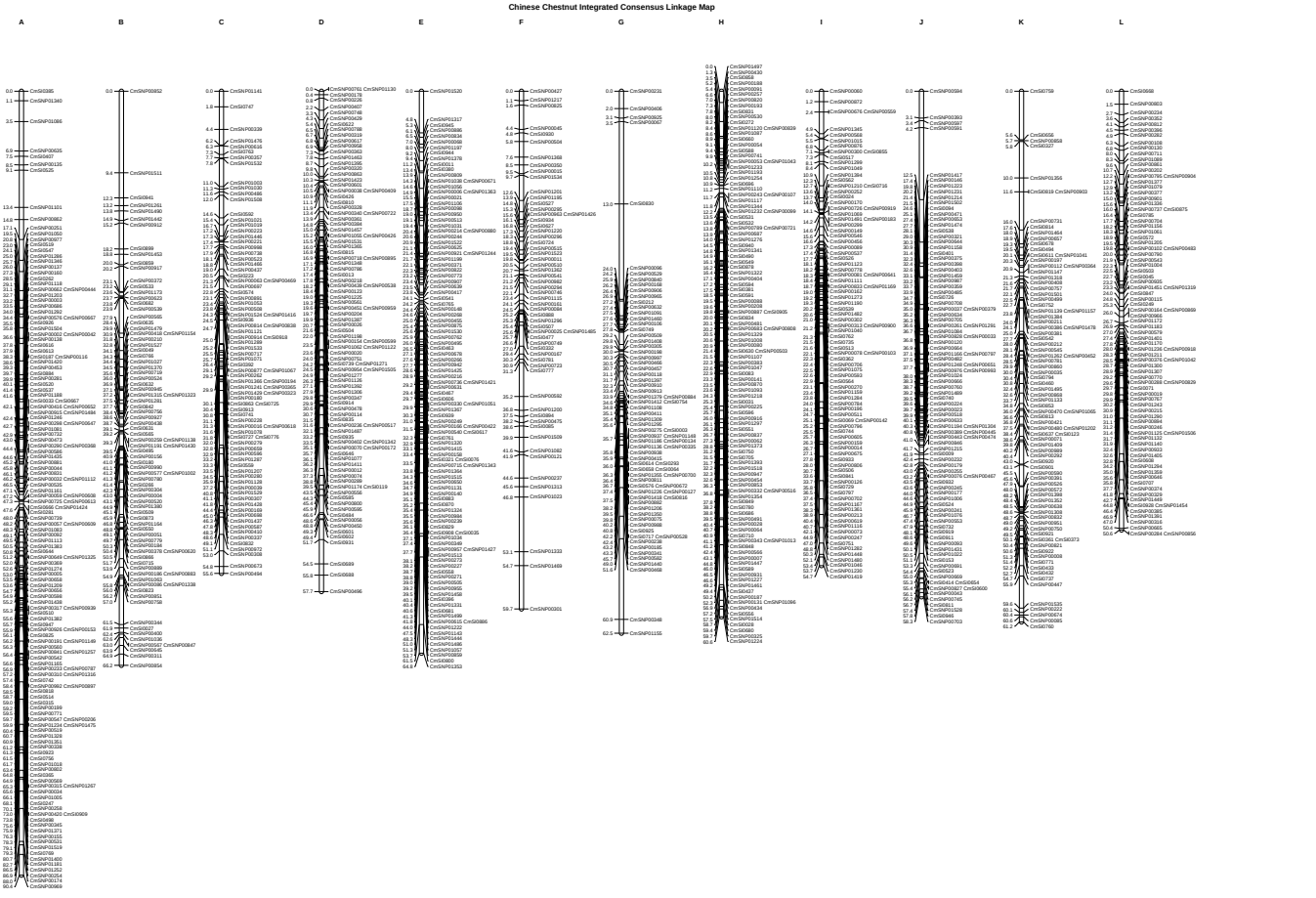
Abbott showed a table of genes that were expressed in much greater levels in Chinese chestnut compared to American chestnut, from the 2009 paper by Ali Barakat. There are different classes of genes that are up-expressed, summarized below.

Disease and defense response genes induced in canker tissues from Chinese and American chestnut.

Hormone signaling	Pathogenesis related	Phenylpropanoid pathway
Allene oxide cyclase	Pathogenesis-related peptide	Cinnamoyl CoA reductase
Oxophytodienoate reductase	Antifungal chitin-binding	Cinnamate-4-hydrolase
ABA 8'-hyrdoxylase activity	Basic chitinase	

Fagaceae Genomics Web. The physical, structural and sequence based information can be found at the website, http://www.fagaceae.org. The physical map can be viewed in either WebFPC or CMap. The process of mapping is developing overlapping very long, extensive pieces of contiguous DNA. Hybridization data from markers and ESTs is also listed.

Genetic mapping and marker analysis. The other part of this project is the overlaying of genetic data over physical data. Many markers are EST-based on genes. The markers are positioned on the physical map as seen on the following linkage map.



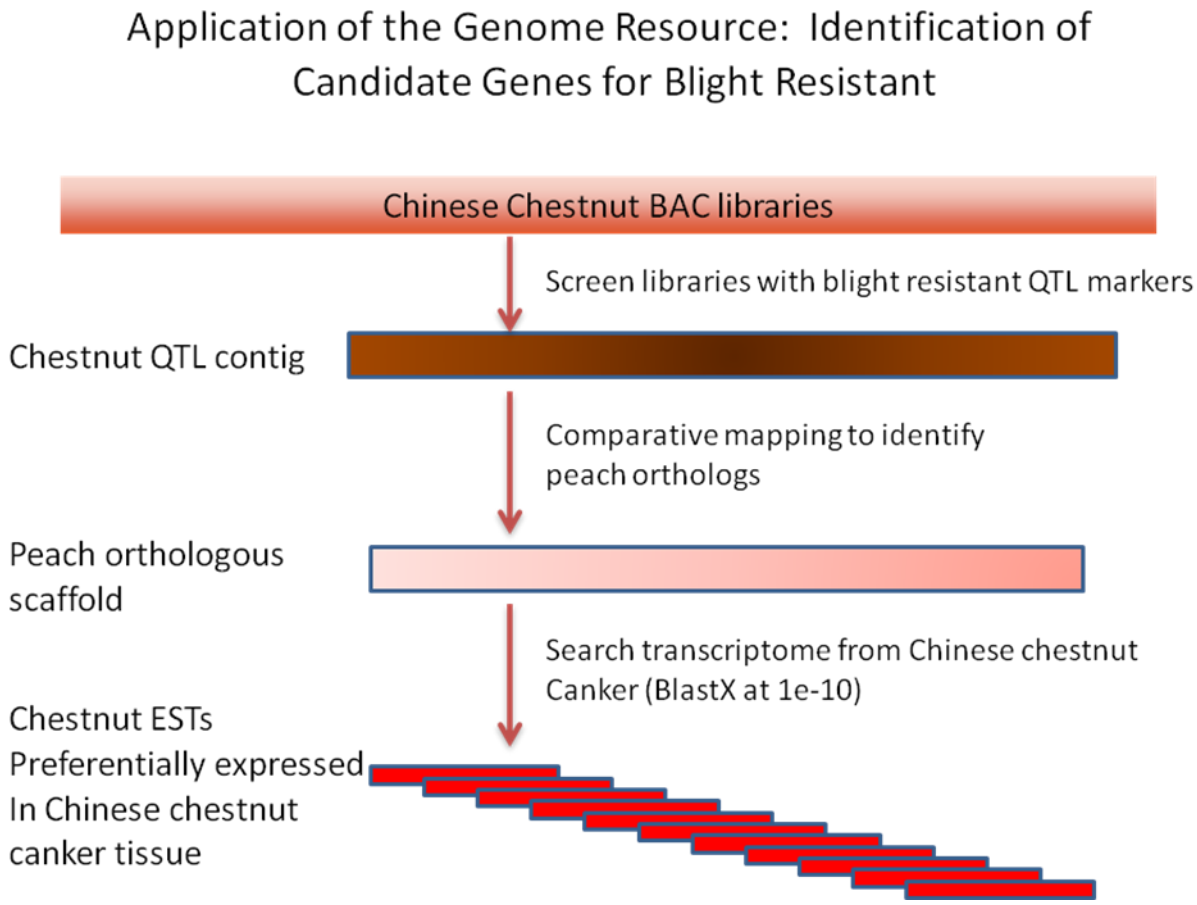
They are in the midst of filling out the American chestnut framework, to be able to go between those maps of information from the Chinese to the American map. Their work flow in the construction of American chestnut framework map is as follows:

- Quick screen for polymorphism (parents and progeny) using touchdown PCR
- Polymorphism markers selected based on

    - SSR producing ≥2 alleles
    - Ample banding pattern
- In event of insufficient markers:
    - SSR with multiple banding patterns, followed by
    - SSR with 1 allele in both parents combined (i.e. strictly dominant markers)

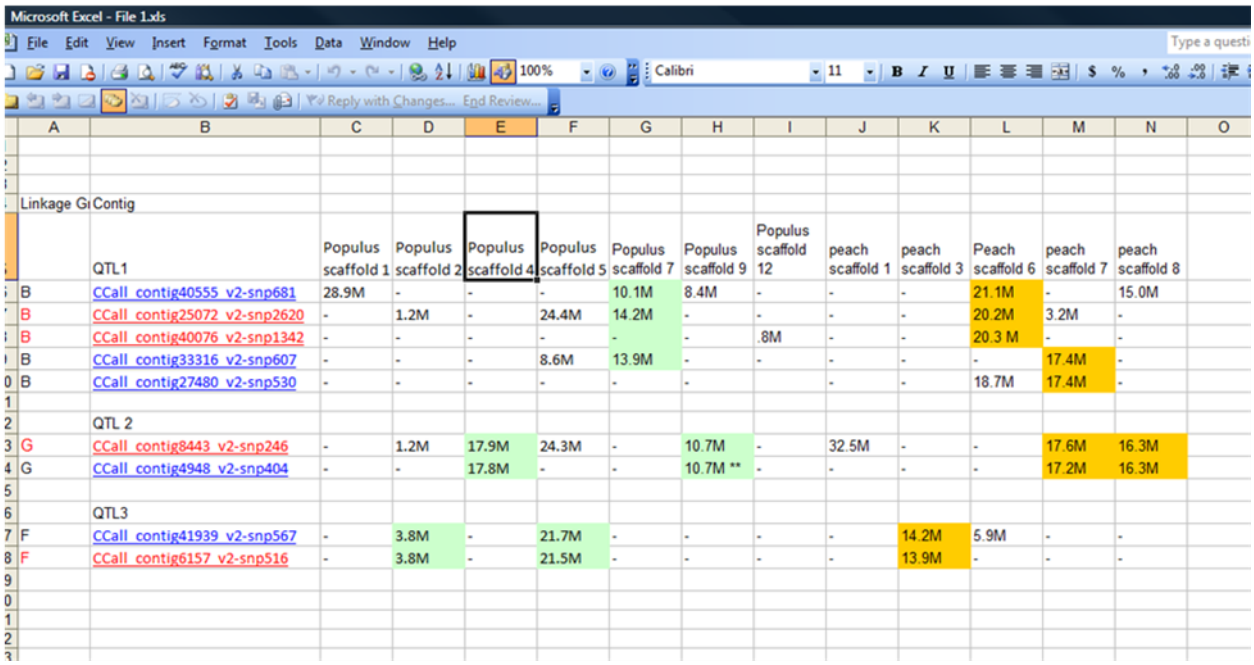
Abbott showed information on linkage group J, taking markers that are in Chinese chestnut and transposing them onto American for the SSR markers. They did a pre-screen of 600 SSR markers, from Tom Kubisiak, and about 50% of them worked reasonably well with one set of conditions. A significant number of the markers will work, so they are now testing for polymorphisms. Abbott expects out of a total of 600 markers, about ¼ of them will be mappable.

Comparative genomics. This is where the rubber meets the road. One of the things they have been pushing for in Abbott's lab is to have a genome sequence for a tree that can be used as a model for gene discovery. That assumes that at some level there is a good preservation of the genome between one species and another. This is not known until it is proven. According to Kubisiak, in a genome-size marker scale, there appear to be large regions that are fairly highly preserved. Abbott stated that he published a series a papers a few years ago looking at EST distribution of peach genome sequences against a number of different species (poplar, etc) and found that there is a quite a good preservation of the peach genome across a number of tree species that falls apart very quickly when looking at something like *Arabidopsis*. He has always had the feeling that having a high quality genome would allow for a jump to other tree species. When the peach genome was finally released publically, Abbott was excited about the opportunity to explore the use of other genomes. In order to do this, you have to be to a certain point—and the point that is critical is that you have to have generated all of the structure that he talked about. It is critical to be able to take markers in chestnut that are genetically mapped that are known to flank the areas of interest, and find those regions in other genomes. The beauty of the way the work was done in chestnut for mapping is the use of expressed genes. All of the markers that have been looked at are either ESTs or SSR or SNPs developed from cDNAs. This allows for the opportunity to cross compare the genomes because the gene sequences are the most highly conserved. If a marker is a gene sequence, it is highly likely that one can find its position in another genome. The strategy is detailed below.



The NSF Project developed structural BAC Chinese chestnut libraries and mapped markers flanking QTLs for blight resistance. The FHI Project will more precisely define these QTLs. We can take all of this sequence information and begin to play with genomes that have been sequenced and ask, "Can I find the markers in one area that are flanking a QTL of blight resistance on another genome?" Abbott's lab uses a lot of bioinformatics tools for peach scaffold sequences and other tree genomes, and looks for regions of synteny between the genomes that overlap. How can one mine 50 or 100 genes on the peach genome to see if they are preserved in American chestnut? The NSF project generated a very deep transcriptome using tissues that were resistant or susceptible in Chinese or American chestnut. There is now a resource of genes that are up- or down-expressed and one can ask, "Are any of these genes found in the QTL intervals in the peach genome?" That was the strategy. Because all of the ESTs and SNPs have been located to the physical map, it is easy to identify those SNPs that define the QTL regions for blight resistance. Abbott presented an example of a SNP marker on linkage group G that was linked to one of the QTLs for blight resistance. His lab was able to identify a contig of Chinese chestnut BAC clones that contained the SNP. This is a start on pulling out the regions of Chinese chestnut that are most closely associated with the QTLs for blight resistance. Abbott has done the same exercise against other genomes; he has taken SNP markers associated with chestnut blight and looked at the poplar and peach genomes. They found regions in these other scaffolds where there are a series of markers, as shown below:

Comparative Mapping of the Blight Resistant QTL Regions in Populus and Peach Genomes



Microsoft Excel - File 1.xls

Linkage G	Contig													
		QTL1	Populus scaffold 1	Populus scaffold 2	Populus scaffold 4	Populus scaffold 5	Populus scaffold 7	Populus scaffold 9	Populus scaffold 12	peach scaffold 1	peach scaffold 3	Peach scaffold 6	peach scaffold 7	peach scaffold 8
B		CCall_contig40555_v2-snp681	28.9M	-	-	-	10.1M	8.4M	-	-	-	21.1M	-	15.0M
B		CCall_contig25072_v2-snp2620	-	1.2M	-	24.4M	14.2M	-	-	-	-	20.2M	3.2M	-
B		CCall_contig40076_v2-snp1342	-	-	-	-	-	-	.8M	-	-	20.3 M	-	-
B		CCall_contig33316_v2-snp607	-	-	-	8.6M	13.9M	-	-	-	-	-	17.4M	-
B		CCall_contig27480_v2-snp530	-	-	-	-	-	-		-	-	18.7M	17.4M	-
		QTL 2												
G		CCall_contig8443_v2-snp246	-	1.2M	17.9M	24.3M	-	10.7M	-	32.5M	-	-	17.6M	16.3M
G		CCall_contig4948_v2-snp404	-	-	17.8M	-	-	10.7M **	-	-	-	-	17.2M	16.3M
		QTL3												
F		CCall_contig41939_v2-snp567	-	3.8M	-	21.7M	-	-	-	-	14.2M	5.9M	-	-
F		CCall_contig6157_v2-snp516	-	3.8M	-	21.5M	-	-	-	-	13.9M	-	-	-

What are the gene sequences in the peach scaffold that are in the region of the blight resistance markers? Abbott is working with Kubisiak to get as high a resolution of the QTL interval as possible. One thing they can do with the peach data is to make connections. Two of the major QTLs for blight resistance in 'Mahogany' chestnut match the loci for powdery mildew resistance in the peach genome (on regions on LG_B and LG_G). It is possible that there is a general pathway between plants and their fungal counterparts.

The QTL regions have a lot of genes that look like they work together. They are all part of a network. A QTL may not be one gene, but a region of genes that are coordinately regulated.

Potential function of ESTs

- Lignin biosynthesis
- Regulation of cellular oxidative status
- Signal transduction for defending pathogens and other biosynthesis pathways.
-

We can use all of these tools and get down from what would be 30,000 genes controlling blight resistance to maybe fifty.

Future direction

- Genetic mapping of the ESTs from the short list to estimate the distance from blight resistance QTLs

- Gene expression profiling of the ESTs by RT-PCR
- Transformation and phenotyping
-

Bloom and Bud Burst dates. Abbott has been working on bloom date and chilling requirements for peach for eight years. In discussing comparative genomics, using chilling requirements in peach as an example, Abbott's lab has identified genes that control chilling requirement. In four different Chinese/American mapping populations, a major locus controlling timing of bud burst in chestnut was localized to Linkage Group L (Hebard, Kubisiak, and Sisco, unpublished data). Kubisiak gave Abbott the SNPs and SSRs that for the bud burst locus, and it turned out that one marker is in the middle of region in peach that Abbott has been studying for years. Abbott showed data from peach, papaya and grape, and there is a lot of preservation of individual genes. Large scale genomes are difficult to align, but when you get down to a fine scale, there is a lot of local preservation of gene order.

Jeanne Romero-Severson, University of Notre Dame

Genetics in chestnut cultivar improvement. The fruits of the Fagaceae genomics project are being used to conduct a translational genetics project in chestnut cultivar improvement. This includes whatever is called chestnut, be it Chinese, American, European, or Japanese. These are the aims of our translational genetics project:

- Cultivar identification
    - One genotype/cultivar
    - One cultivar/genotype
- Cultivar pedigree
    - Interspecific cross
    - If yes, which species
- Cultivar relationship
- Germplasm characterization
- Identification of genetic diversity and differentiation in native *Castanea*

The first aim is to straighten out the mess of the cultivars, because there is always a mess. Second, to ascertain the cultivar pedigree. This is important because it gives you some ability to predict what one cultivar will do in respect to another cultivar. If there is a possibility of an interspecific cross (and with chestnut there are at least four species), you might want to know that: (1) this might influence who the good pollinator is (one reason for poor pollination is that the pollinator may not be quite right for the interspecific cross); and, (2) which species is in this interspecific pedigree. Lastly, once more information is obtained, one can do germplasm characterization. For us, that means developing genetic fingerprints for all cultivars, regardless of species and identifying the genetic relationships among all cultivars. Next, we need to do a rangewide diversity and differentiation project for all *Castanea* species native to the United States. There was an historic project done by Tom Kubisiak and colleagues. That was done with the tools that were available at the time, using isozymes and RAPDs. Isozymes have too few polymorphisms for fine-scale differentiation studies and RAPDs may not perform well where species boundaries are weak. The study needs to be repeated with current tools.

"What cultivar is this?" is a genetics question. If one genotypes six cultivars of the same name and they are all different, what is the cultivar? Romero-Severson uses the majority rules process—if

five things are the same with 95% certainty, then it might be defined as 'Colossal' and the others are variants of 'Colossal'. What does "cultivar" imply? Are all cultivars with the same name the same? (Never). Are all cultivars with different names different? (of course not). Establishing identity is the first step in cultivar improvement.

Why does this matter? It matters because you begin to be interested in the expression of the phenotype across the landscape, whether you are working with natural populations or cultivars. If you are working with cultivars, you are interested in performance (nut quality, disease resistance, stress resistance). It is important to know if one grower is working with 'Colossal' and 'Peach' that those same cultivars identified as 'Colossal' and 'Peach' in another orchard are the same genotypes, so that inferences can be made about the influence of the genotype in the environment in which they are growing. 'What is the pedigree?' is a genetics question. We have the right to assume that all grafts from the same tree are the same. If they are not, we have the right to assume that something happened. We know that the seed on a named cultivar is not that cultivar. This is not obvious to all the chestnut hobbyists. You often get, what is clearly, a half-sib to a parent tree. All of the seeds of a given tree are different. The amateurs do not always know this, and they may not fully grasp genetic principles. Next, A X B is not the same as B X A. The chloroplasts and mitochondria can differ. The fertility can differ. Even the word "hybrid" means different things to hobbyists, plant breeders and botanists. This can create endless confusion.

Once we have identities straight, we can examine combining ability. Some crosses work very well and you get good progeny. Other crosses do not yield good outcomes. Superior hybrids may have an inferior parent. One cannot always tell by parental performance. Dominant traits are easy to see. Recessive traits are not as easy to distinguish. Also, the best traits are usually determined by the many genes, not simple Mendelian single genes.

Chestnut is complicated because it has at least four species. There are many years of undocumented cultivar development. The source of germplasm is not always known. With the right tools, we can tell which cultivars are hybrids, the species identity of both parents and in some cases the parents themselves. Chestnuts are diploid; this is straight-forward. We also can acertain the genetic basis of a phenotype of interest but that is not so simple.

What have we already done for chestnut? The Romero-Severson lab, in collaboration with the Center for Agroforesty at the University of Missouri has developed a set of robust EST/SSR markers from sequences posted on the Fagaceae Genomics website. Most of the 30 markers tested are polymorphic. Using the best 12 of these markers, we generated genotypes for 296 chestnut cultivars (individual trees) and detected 10 major genetic groups from the 60 groups we expected based on cultivar names. This was determined by genotyping on a 3730 XL capillary electrophoresis machine and doing a Bayesian analysis (STRUCTURE) on the result. As an example of the many cultivar misidentifications found, Romero-Severson found two entries named, 'Colossal' that were quite different form the other Colossal entries, which were identical to each other. That does not mean that they will look different but her analysis indicates that they are different. She feels that their performance may be different. She also showed analyses of the cultivar 'Peach', a cultivar recommended by the Agroforestry group at the University of Missouri. All of the tissue samples were taken from trees at the New Franklin orchard at the University of Missouri. Many of the 'Peach' cultivars were identical, but there were a few that were different, as indicated by the same Bayesian analysis.

Unlike the nuclear genome, the chloroplast is rock-solid. The chloroplast test is simple and inexpensive, so there is no excuse for not knowing what chloroplast you have in your cultivar or germplasm. She would like to develop a small set of markers to distinguish chlorotypes and at least 30 nuclear markers that work across all species. By work she means that they amplify, they are robust and trustworthy and they are in real genes. She would like to establish a genetic database for source species. She also would like to identify species ancestry.

In looking at other forest tree populations, Romero-Severson found that the geographic distance between populations is a very poor predictor of genetic distance between the populations, especially in the north (north of the last glacial maximum). She feels that it is wise to really make an effort to sample the edge populations in the north and south, in populations that are gradually dying out, and to use the marker systems that we have currently. This has scientific value and it has value to restoration. If you want to incorporate frost tolerance, you should know if a northern tree is genetically different from a chestnut 500 km to the south. Chestnut, butternut and oak have been pushed south four times in the last 800,000 years. There have been four extensions of the ice sheet and each time a species has to repopulate back north. Population genetics predicts that there will be differentiation the further north a species migrates. This differentiation may be good for the species but one must recognize that differences may occur and sampling must take this into account.

Within 3-4 years, the cultivars in our existing collections can be genotyped and thoroughly characterized. We do not have a good representation of European chestnut but perhaps we can obtain samples from European colleagues. A foolproof species-specific detection kit can be developed for chloroplasts. Also, if funded is secured, she can develop a robust set of nuclear markers for the following aims:

- Assign species
- Hybrid detection and parent species assignment
- Cultivar definition and detailed cultivar relationships
- Genetic diversity and differentiation for *C. dentata* and the sympatric chinkapins

Romero-Severson also hopes to associate pedigrees with certain traits.

Fred Hebard, The American Chestnut Foundation®

Sclerified phelloderm. Hebard posed the question, 'are we capturing the comparison of resistance with the basic screening for blight resistance?' He inoculates trees in the late spring and rates them in the late summer/early winter and again the following spring. He has been working with sclerified phelloderm for many years. In 1982, Chinese chestnut had the characteristic of sclerofied phelloderm and American chestnut did not. Sclerification correlates with resistance measurements in backcross trees. Hebard feels that selection for resistance traits will inherently be a selection for sclerification. When Hebard first looked at sclerification, all American trees did not have it, and all Chinese chestnut did. When he began looking more closely, some American chestnut trees had some sclerification while some Chinese chestnut had none. This may be controlled by a single gene. Thus, sclerification does not distinguish the two species. It appears that the TACF® breeding program is selecting for sclerification as sclerofied phelloderm appears in the backcross trees. In segregating progeny, it fits 1:1 but his analysis still leaves some questions. The bottom line is that it may be a simply controlled trait.

William MacDonald, West Virginia University

Backcross orchard for assessment of host resistance combined with hypovirulence W.L. MacDonald and M.L. Double (in cooperation with Fred Hebard, Bob Paris and Sara Fitzsimmons, The American Chestnut Foundation®). Six replicate plots containing 150 trees each were established at the Plant and Soil Sciences Farm in Morgantown, WV in the spring of 2005 for the purpose of assessing the interaction of host resistance and virulent/hypovirulent strains of *Cryphonectria parasitica*. Seeds have been replanted annually from 2006-2010. As of August 2010, survival is as follows:

Species	Percent Survival
American	71%
Backcross	80%
Chinese	91%
European	46%

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

Paul Sisco, TACF®, Asheville, NC

Cytoplasmic male sterility. Cytoplasmic male-sterility (CMS) has been studied in many plant species. It is often found in the progeny of interspecific hybrids, caused by an incompatibility between nuclear and mitochondrial genotype. (C.D. Chase, Trends in Genetics 23(2):81-90, 2007). Shi and Hebard reported on CMS in Chinese / American chestnut hybrids (J. Amer. Chestnut Foundation 11(1):38-47, 1997). In most cases studied so far, F_1 hybrids of Chinese and American chestnut are male-sterile when the Chinese nuclear genome is present in the American cytoplasm, but all other combinations of nucleus and cytoplasm are male-fertile (Fig. 1). Data from Shi and Hebard, plus additional data from a 'Nanking' BC_1 mapping population (Hebard and Sisco, unpublished) indicated that the sterility could be dependent on a single gene from Chinese chestnut.

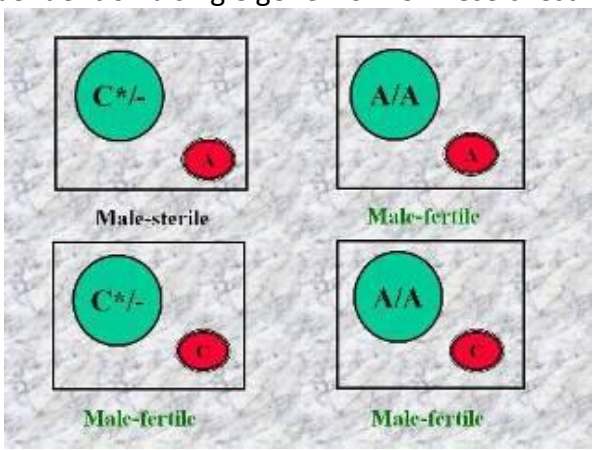


Fig. 1. Pollen phenotype of the four combinations of Chinese or American nuclear genome with Chinese or American cytoplasm.

In chestnut, as in most other plants, chloroplasts and mitochondria are maternally inherited. The male-sterile phenotype can be hidden for generations if one keeps the crosses in Chinese cytoplasm, because in Chinese cytoplasm the trees will always be male fertile. As soon as American cytoplasm is introduced, sterility will show up if the Chinese gene for male sterility is still present in the nuclear genome. This is what happened at Meadowview. The first two crosses to create the 'Clapper'

and 'Graves' BC_1 trees were done in such a way that both these trees were in Chinese cytoplasm. When Fred Hebard brought pollen from 'Clapper' and 'Graves' to Virginia and pollinated American trees, the resulting BC_2 progeny were in American cytoplasm for the first time and male-sterility showed up.

But exceptions to this schema have been reported by Hill Craddock and others who have made F_1 hybrids using American chestnut trees from the South. These hybrids, even when in American cytoplasm, are male fertile. Sisco thinks there is an explanation. Hill Craddock and his colleague Joey Shaw at the University of Tennessee at Chattanooga have found different cytoplasm types in Castanea species in the southern US, based on chloroplast genotype. One chloroplast genotype (*P*) is found primarily in Allegheny chinquapin, one (*O*) in Ozark chinquapin, one (*D*) in most American chestnut and a fourth (*M*) in American chestnut in North Georgia and Middle Tennessee, which in terms of chloroplast genotype, is distinctly different from the other three. Many of the American chestnuts found in this region are in *M* cytoplasm. So far, every male fertile F_1 hybrid in American cytoplasm has the *M* chloroplast type. the correlation is perfect. Since mitochondria and chloroplasts are inherited together, Sisco thinks the atypical male fertility may actually be dependent upon the mitochondria associated with the M chloroplast type. To further test this hypothesis, Joey Shaw and Tom Kubisiak are going to determine the chloroplast genotypes of the 'Musick' and 'Mill Creek H' American chestnut trees that were used as female parents of a 'Nanking' BC_1 mapping population. The BC_1 trees that had 'Musick' as their female parent were all male-fertile, whereas the BC_1 trees that had 'Mill Creek H' as their female parent segregated 50/50 male fertile/sterile. The hypothesis is that 'Mill Creek H' has the *D* chloroplast type whereas the 'Musick' tree had one of the other chloroplast types identified by Joey Shaw (Systematic Botany, in review).

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*

Brad Hillman, Rutgers University

Hillman, Senior Associate Director for the New Jersey Agriculture Experiment Station and Director of Cooperative Research, has acted as the Administrative Advisor for NE-1033 for the past three years. In 2008, Hillman nominated NE-1033 for a regional award, and NE-1033 was recognized as the 2008 winner. As a result, NE-1033 was automatically put in the running for the national award (which it did not receive). In 2010 at the NERA meetings, it was suggested that NE-1033 be nominated again for the national award. Hillman indicated that Don Rossi, director of NERA administration, deserves a lot of credit as NE-1033 was honored with the national award in 2010. Hillman indicated that this is a great achievement for NE-1033 as there are approximately 150 regional projects around the country. The award will be presented at the APLU (Association of Public Land Grant Universities) meeting in Dallas in November. With this award comes with $15,000; the group gets to determine how the money will be spent. It can be used to send representatives to the APLU meeting or symposium travel or start-up money for the 2010 international meeting. Hillman was not sure of the timeline for when the money has to be spent. Hillman encouraged those at land grant institutions to thank their Experiment Station Directors.

Reovirus update. Hillman is finishing the sequence analysis of a second *Cryphonectria* Reovirus.

Phytophthora update. Hillman's research associate is working on *Phytophthora infestans* viruses; they would like to move information to other *Phytophthora* species.

Eastern filbert blight. Hillman, in conjunction with others, is working on a draft of the genome of the fungus that causes Eastern Filbert Blight, *Anisogramma anomola*. It is an unimportant parasite causing a small canker on the native American Hazelnut, *Corylus americana*. However, it is lethal on the introduced and commercially important European Hazelnut, *Corylus avellana*.

Community bioinformatics. Hillman began talking with Angus Dawe, three years ago, about a community sequencing project. They would like to get community involvement for this project. There has been a lot of nice individual work done in specific locations on fungal isolates and viruses from fungal isolates but there has not been a whole N. American coverage. With new DNA sequencing technologies, now seems to be the time to do a large number of genome sequences of viruses from all over the country. They both see this as developing from being primarily a scientific-based to a community/outreach/learning/extension/education program. Hillman has in mind that couple of base centers which are doing bioinformatics at major research institutions and they would feed out to other universities and 4-year colleges and even to high schools: selection of isolates; GPS mapping where isolates came from; examination of trees; subculturing isolates; and, nucleic acid extractions, etc. All of this information would ultimately feed into a centralized sequencing facility and then bioinformatics would flow down from the university level back down to the high school level. There has been enough progress made at the fungal level that a whole lot of genotyping of the fungus can be done at the same time. A lot of information on trees also could be done at the same time, in conjunction with georeferencing. This would give us the opportunity to put a nice story together that has broad coverage that would make for a good evolutionary biology story. At the heart is an education/outreach project to provide hands-on teaching tools. Centralized training sessions can then be offered to present organized information. This will require a buy-in from many people. He hopes to write an NSF grant with the hope that information can encompass a number of scientific levels and get back to mainstream users. The key to funding is to go from a descriptive level to a hypothesis level.

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

DOE/JGI Community Sequencing Program: Project Proposal. *Project Title:* Genome sequencing of the chestnut blight fungus *Cryphonectria parasitica* (submitted March, 2006, approved June, 2006).

Proposer's Name:
Donald L. Nuss, Center for Biosystems Research, University of Maryland Biotechnology Institute

Other Proposers:
Alice C. L. Churchill, Department of Plant Pathology, Cornell University
Michael G. Milgroom, Department of Plant Pathology, Cornell University

Other participants:
John Carlson, Pennsylvania State University, USA

Baoshan Chen, Guangxi University, PR China
Angus Dawe, New Mexico State University, USA
Bradley Hillman, Rutgers University, USA
Dae-Hyuk Kim, Chonbuk National University, South Korea
Thomas Kubisiak, USDA Forest Service, USA
Myron Smith, Carleton University, Canada
Neal Van Alfen, University of California, Davis, USA
Michael Wingfield, University of Pretoria, South Africa

Abstract:

Cryphonectria parasitica, the chestnut blight fungus, is responsible for epidemics that caused the destruction of tens of millions of mature chestnut trees in forests of North America and Europe during the first part of the 20th century. The discovery of a group of RNA viruses, now classified in the family Hypoviridae (hypoviruses), that reduce the virulence (hypovirulence) of this pathogen stimulated intensive research into the potential of using fungal viruses for the biological control of fungal disease. Subsequent epidemiologic and population genetic studies have established the chestnut/*C. parasitica*/hypovirus pathosystem as the textbook example of both the consequences of accidental introduction of an exotic organism and of hypovirulence-mediated biological control of fungal pathogens.

Interest in *C. parasitica*, hypoviruses and their interactions now extends well beyond disease control potential. The development of a robust *C. parasitica* transformation protocol and of hypovirus reverse genetics led to the establishment of a biologically relevant experimental system with the rare capacity for efficiently manipulating the genomes of both a eukaryotic virus and its host. Scientists studying this system have also made significant contributions to the current understanding of mycovirus-host interactions, fungal population genetics, mechanisms underlying fungal pathogenesis and fungal signal transduction pathways. Very recent advances with this system are providing important new insights into the role of RNA silencing as an antiviral defense mechanism in fungi and the impact of viruses on fungal vegetative incompatibility systems and secondary metabolism. Thus, availability of the *C. parasitica* genome sequence, the first for an Ascomycete tree pathogen, would greatly accelerate the efforts of an active and growing research community that address a broad range of important fundamental and applied research topics.

The objectives of the proposal are to: A) assemble an 8-10 X sequence coverage for the genome of *C. parasitica* strain EP155; B) perform an automated and directed annotation of the assembled genome sequence; and C) provide a web-accessible database of the *C. parasitica* genome sequence with necessary tools for mining and comparative genome studies by the research community.

Scope of Work:

Genomic DNA will be prepared from the most widely used and best characterized *C. parasitica* strain, Ep155. Expectations from JGI include the generation of approximately 400Mb of raw sequencing reads required for 8 – 10 X coverage of the estimated 40-Mb *C. parasitica* genome, sequence assembly and initial automated annotations. We also requested assistance in organizing a gene annotation jamboree with members of the *C. parasitica* research community. The annotation process was aided by the availability of two large *C. parasitica* EST libraries that have been generated by laboratories in the U. S. and China and a linkage map; annotation was aided further by comparisons

with the completed genomes of the phylogenetically related fungi *Neurospora crassa* and *Magnaporthe grisea*.

Update on JGI Genome Sequencing Project: The JGI released version V2 of the *C. parasitica* genome assembly to the public in March 2010. The assembly release of whole genome shotgun reads was constructed with the Arachne assembler and improved with finishing reads. This release contains 26 main genome scaffolds totaling 43.9 Mb. Five scaffolds are considered complete telomere-to-telomere and an additional six having a telomere at one end. The remaining 15 scaffolds are smaller and do not contain telomeres. Roughly have of the genome is contained in four scaffolds all at least 15 Pbp in length.

Nuclear Genome Assembly	**V2.0**
Scaffold count	26
All contig count	33
Scaffold sequence bases total	43.9 Mb
Scaffold (large) contig sequence bases total	43.8 Mb
Estimated % sequence bases in gaps	0.2%
Scaffold N50/L50	4/5.1 Mb
Contig N50/L50	5/4.0 Mb
Number of scaffolds > 50 Kb	13
% in scaffolds > 50 Kb	99.6%

Annotation. Annotation of the v2.0 assembly was produced by JGI Annotation Pipeline using a variety of homology-based and *ab initio* predictors. The v1.0 Gene Catalog and its manual curations were also mapped to the v2.0 assembly and were included in the filtering procedure that determined the initial v2.0 Gene Catalog. After filtering EST support, completeness and homology support, a total of 11,609 genes were structurally and functionally annotated.

Nuclear–Genome Annotation	**V2.0**
# gene models	11,609
Gene density (genes/Mb scaffold)	264.44
Average gene length	1648.61
Average protein length	422,99
Average exon frequency	2.91 exons/gene
Average exon length	487.09
Average intron length	122.58
% complete gene models (with start/stop codons)	84%
% genes with homology support	85%
% genes with Pfam domains	66%

Nuss reported that a paper is being put together. All of the information is there; he is waiting on contributions from authors and it will be submitted. There are a number of chromosomes that are completely sequenced and telomere identified and conclusions can be made that will tie together the genetic linkage map, the sequencing and the karyotyping. There is an emphasis to get the genome paper published because there are additional papers that are forthcoming that will rely on the initial genome publication.

Re-sequencing of the genome of *C. parasitica* strain Ep146. At the NE-1033 meeting in Michigan, there was discussion as to what additional isolates of *Cryphonectria parasitica* should be sequenced. Nuss was interested in Ep146, a brown-pigmented isolate, because he felt it could be used to go after some of the vegetative incompatibility genes because Ep146 is of the opposite mating type from Ep155, the reference genome. When these two isolates are crossed, the ascospores segregate into brown and orange ascospores. He had shown previously that these two isolates differ at multiple *vic* loci. Nuss felt if Ep146 was sequenced and that data laid over the sequence of Ep155, there would be heterogeneity in the places where the *vic* genes reside. Differing *vic* genes cause programmed cell death and virus transmission is blocked. He is interested in knowing what the genes are that control vegetative compatibility. While Angus Dawe is focused on the downstream genes, Nuss reported on the upstream genes. They made use of the 64 European Union tester strains provided by Michael Milgroom that have been tested at each of the *vic* loci. Each isolate has either allele one or two at each locus. By testing isolates against all 64 tester strains, EP146 was found to differ from EP155 at four *vic* loci. Ep146 DNA was prepared and sent to the New Mexico State University Sequencing Center where ~11X coverage (486.6Mbp) was sequenced using 454 technology. Raw reads were individually mapped onto 39 scaffolds comparing the version 1 Ep155 reference genome sequence within a searchable genome browser and independently assembled into 3600 contigs. A browser was set up which was key to the comparison. Candidate genes have been identified for alleles of *vic2*, *vic4*, *vic6* and *vic7*. Disruption of one candidate *vic* allele for a pair of heteroallelic strains that differ at *vic2*, *vic6* and *vic7* did not eliminate barrage formation. Disruption of one candidate *vic* allele for a pair of heteroallelic strains that differ at *vic2*, *vic6* and *vic7* did increase virus transmission to the mutant strain. Disruption of both candidate alleles for a pair of heteroallelic strains that differed at *vic6* resulted in the elimination of barrage formation. Detailed characterization of the candidate *vic* gene alleles is well under way.

Angus Dawe, New Mexico State University

Post-translational modification of phosducin like protein BDM-1. Phosducin-like proteins are conserved regulatory components of G-protein signaling pathways, which mediate many physiological processes. Identified throughout eukaryotic genomes, they are thought to serve as regulators of Gβγ assembly. *Cryphonectria parasitica* contains three Gα, one Gβ, one Gγ subunits and phosducin-like protein BDM-1 that have important roles in pigmentation, sporulation and virulence. Deletion of either Gβ subunit or BDM-1 produces identical phenotypes. Additionally, we report that the Gβ subunit is not detectable in absence of BDM-1. Given that the regulatory role of phosducin-like proteins may be influenced by protein kinase II (CK2), we confirmed that BDM-1 is a phosphoprotein that can be targeted by CK2 *in vitro*. Mutagenesis of the five putative CK2 sites revealed that native phosphorylation likely occurs at two locations. Strains bearing a single or double serine to alanine substitutions at those sites were significantly less virulent with only minor phenotypic changes from vegetative colonies. Therefore, CK2 activity appears to mediate key signals that are required for virulence, but not for vegetative growth. Expression of selected CK2 mutants resulted in reduced accumulation of the Gβ subunit, suggesting that phosphorylation of BDM-1 influences Gβ stability.

Genome Sequencing for the purpose of identifying *vic* loci. Strain EP146 was fully sequenced to approximately 10-11X coverage using the 454 sequencing facility at NMSU. Performed in collaboration with Don Nuss, the sequencing generated:
Filter Passed Reads: 1,219,139
Total Bases: 468,614,713 bp
Ave. Length: 384.38
Median Length: 431.0
See Maryland report for further details.

Identification of *vib-1* as a potential regulator of the vegetative incompatibility response. The *vib*-1 gene has been previously identified in *Neurospora crassa* as a potential mediator of programmed cell death, with implications for the transmission of signals relating to vegetative incompatibility. In the interests of identifying components with potential for influencing hypovirus transfer between strains and thus the potential for biological control, a candidate *vib-1*-like gene was identified in the *C. parasitica* genome sequence. A knock-out construct has been generated and used to delete this gene from the EP155 strain. This has resulted in a phenotype that closely matches the predictions derived from the *N. crassa* data with increased sporulation but greatly diminished pathogenicity. Removal of *Cpvib-1* does not, however, impact barrage formation between incompatible strains when only one strain lacks the gene, suggesting that this protein product can be provided *in trans*. However, we have also removed *Cpvib-1* from a second, incompatible, strain, EU1. In this case, when paired with Δ-*Cpvib-1* (EP155 background) the normal barrage formation is absent and the strains appear perfectly compatible. Furthermore, an analysis of compatible and incompatible hyphal interactions has shown that the level of cell death is reduced in the absence of *Cpvib-1* (Figure 4). Therefore, *Cpvib-1* does play a role in signaling the onset of the incompatibility response, including cell death, for at least a subset of the triggers induced by mismatched *vic* loci. Further gene deletions are in progress using the EU tester strains (Cortesi and Milgroom, 1998; Appl Environ Microbiol *64*, 2988-2994) to determine which of the *vic* loci signal the incompatibility response through the *Cpvib*-mediated pathway.

Debora Jacob-Wilk, UC Davis

***In silico* analysis of the *Cryphonectria parasitica* secretome for Kex2 processed sequences and expression analysis of its nine putative Kex2 processed, secreted aspartic proteases.** Jacob-Wilk is interested in how hypoviruses affect the fungal secretory pathway. It was shown previously that hypoviruses causes vesicle accumulation in the fungus and these vesicles are used for hypovirus replication. Also several of the hypoviral elements were found to co-localize with trans-golgi elements of the fungus such as Kex-2 of AP-1μ. Previous work showed that several genes that were being down-regulated by the hypovirus contain Kex-2 processing led them to study the role of Kex-2 in fungal pathogenicity. When Kex-2 was silenced, they found that *C. parasitica* pathogenicity was significantly diminished. She wants to investigate what proteins are found at the fungal-host interface. Her analysis began with 11,184 ORFs; of those, approximately 1,299 had signal peptides. This means that they were going to the secretory pathway, but not all proteins that go to the secretory pathway are actually excreted. Some go there for post-transcriptional modification. The analytic process:

Aspartic Proteases		
Prot. ID	**Homologies and similarities**	**Domain homologies**
90510	Pepsin-like	Peptidase A1 Apartic active site
34127	Pepsin-like	Peptidase A1 Apartic active site
87438	Pepsin-like	Peptidase A1 Apartic active site
37154	Pepsin-like	Peptidase A1 Apartic active site
71039	Pepsin-like	Peptidase A1 Apartic active site
43429	Aspartic type endopeptidase	Peptidase A1, ABC transporter
107093	Aspartic protease	Peptidase A1 Aspartic protease
62389	Endothiapepsin	Aspartic protease precursor
38462	Endothiapepsin	Aspartic peptidas active site

One-hundred and fourteen ORFs were identified based on sequence homology to other known genes and fell into three major protein groups. They included cell wall modifying enzymes, extracellular proteases and oxidoreductases. Also identified by the screen were kinases, lipases, esterases and transporters.

Nine aspartic acid proteases were chosen for further genetic characterization. Expression of these genes was studied in wild-type and CHV-1 infected strains grown in liquid media and liquid media supplemented with chestnut wood.

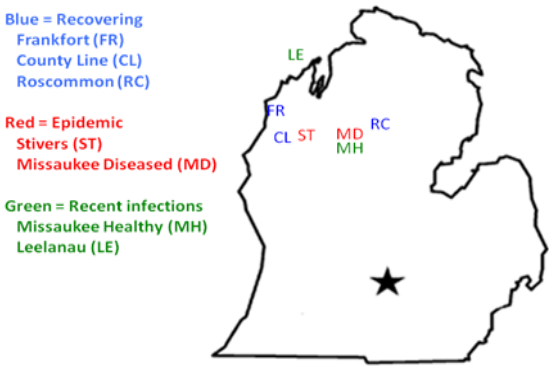
- Two of the proteases transcripts, 71039 and 43429, were detected at significant levels in both wild-type and CHV-1 infected strains. No significant differences could be detected in expression levels with or without wood supplement in liquid media.
- In the wild-type strain, transcript levels of three proteases, 38462, 34127 and 107093 were significantly enhanced when grown in the presence of wood. No significant change in transcript level was detected in the presence of CHV-1.
- Two proteases, 90510 and 37154 were highly expressed in the wild-type strain grown without wood supplemented media, but were significantly down-regulated in the presence of wood. Infection by CHV-1 did not change expression.
- Two proteases 62389 and 87438 were up-regulated in the presence of wood, but down-regulated in the presence of CHV-1.

Further characterization of aspartic proteases 71039, 90510, 62389 and 87437 was done using reverse genetics. The results showed that individual loss of any of these four proteases did not significantly affect growth rate, conidogenesis or virulence of *C. parasitica* under the conditions tested.

Josh Springer, Michigan State University

Hypovirus inoculations in three Michigan blighted populations. Springer used matrix demography to deploy GH2-containing hypoviruses to 1-10 cm American chestnut trees. He did not attempt to save the largest trees. Isolates from existing cankers were converted to hypovirulent and he measures effective spread of hypoviruses between trees. Initial treatments were in June/July 2009 using a drill to create holes for the hypovirus slurry. Cankers were sampled and retreated in May 2010. Springer will continue annual sampling of treated and non-treated trees. He will test for the presence of hypovirus using gel electrophoresis rather than simply relying on phenotype.

Vegetative incompatibility diversity in Michigan populations of the chestnut blight fungus, *Cryphonectria parasitica.* Initial isolate collections were made in the 1990s by Anita Davelos. Those sites included: recovering sites (Frankfort, County Line and Roscommon); and, epidemic sites (Stivers and Missaukee diseased). Two additional sites were added in 2009 by Springer (Missaukee healthy and Leelanau). Those sites are identified below:



To obtain *C. parasitica* isolates, bark samples were sterilized, plated onto PDA and the cultures were single-spored to obtain hypovirus-free isolates. All isolates were paired against each other in triplicate. Compatibility tests were conducted on chestnut wood imbedded in PDA (bark is removed from chestnut twigs, autoclaved and imbedded in media—seven per plate). Plates are photographed to keep a record of reactions. Data of the change in vc populations is listed in the following table.

Population	N	VC Groups
County Line 1996	51	2
County Line 2009	30	2
Roscommon 1996	40	2
Roscommon 2009	30	1
Frankfort 1996	38	4
Frankfort 2009	30	8
Stivers 1996	30	9
Stivers 2009	30	11
Missaukee Diseased 1996	20	4
Missaukee Diseased 2009	30	4
Missaukee Healthy 2009	30	6
Leelanau 2009	30	6

The population structure has not changed much at County Line and Roscommon between 1996 and 2009. The number of vc types at Frankfort has doubled from 1996 to 2009; four new vc groups were detected in 2009. Perhaps, not coincidentally, the trees at this site have been in decline over the past 5 years. Vegetative compatibility diversity at the four sites without hypovirus was generally higher than that found at the three recovering sites. Further, common vc groups were often shared among sites, but surprisingly, distance between sites was not correlated with the sharing of vc groups.

Andrew Jarosz, Michigan State University

Hypovirulent inoculation study at West Salem, WI. This study was designed to document the degree to which hypoviruses ameliorate the symptoms of chestnut blight on trees at West Salem. One-hundred, sixty-four trees along the epidemic front were chosen for study in 2006 and assigned to one of two groups. Euro 7 was inoculated onto 118 trees (at the base and at 6') and applied annually. Forty-six trees were designated as disease controls and disease was allowed to develop naturally. Hypovirulent inoculations continued into a fifth year for trees in the hv group. Main stems of trees in both treatments continue to die back. As of June 2010, 61% of the control trees have died back and 35% of the hv treated trees have died back. Crown ratings of surviving trees are only marginally better for the hv group, but there is a higher percentage remaining. Of the trees initially assigned in 2006, a larger percentage of trees from the hv group are still rated 1 or 2 in 2010 (crown rating 1=live crown; 2=dying crown; and 3=dead crown but epicormics are present). For hypovirus-treated trees, 21.2% of the trees are rated 1 or 2 compared to 6.5% of the control trees. The remaining questions include:

- Will hypovirus treatments simply provide a 2-3 year delay before the main stems die or will a proportion of these stems actually survive?
- If the main stems survive in the hypovirus treatment, will the crowns eventually begin to rebuild?

Mark Double, West Virginia University

Introduction of hypoviruses at West Salem, Wisconsin W.L. MacDonald and M.L. Double (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 2010. Two methods were employed to evaluate whether biological control had been initiated: (1) punch treatment of individual cankers as done initially; and (2) wounding of trees by inoculating scratch wounds to the bark surface to create reservoirs of inoculum. One-half of the plots in each region were punch treated and the remaining half were scratch-treated. Approximately 25% of the trees in each plot were untreated to assess tree-to-tree spread.

Hypovirus spread has been assessed annually by analyzing isolates of *C. parasitica* that arise from bark samples taken annually from cankers. Hypoviruses are recovered most readily from treated cankers followed by non-treated cankers on treated trees. Hypoviruses have spread less effectively to non-treated trees. This is the case regardless of treatment method.

In June 2010, a study examined the spread of hypoviruses to American chestnut trees outside the permanent plots (similar trees were examined in 2008). Bark samples were removed from cankers on trees in a 15m zone beyond the perimeter of each plot. The bark samples were processed and the resulting colonies identified for presence or absence of hypovirus. Bark samples were taken from 345 cankers on 225 trees. Only 30% of the bark plugs have been analyzed. To date, hypovirulent isolates have been recovered from 80% of the trees on the perimeter of the 'Disease Center' plots (an area

with the longest treatment history), 16% from of the trees outside the 'Front' plots; and 8% from perimeter trees in 'Beyond the Front' plots (the most recently infected portion of the stand). Data are similar to that of 2008 samples of similar trees.

The biological control potential of *Cryphonectria parasitica* strains containing an infectious cDNA copy of the hypovirus CHV1-Euro7 W.R. Rittenour, M.L Double, W.L. MacDonald (in cooperation with D.L. Nuss-University of Maryland Biotechnology Institute)

This study, initiated in 2004, was designed to evaluate whether transgenic *C. parasitica* strains containing a cDNA transgene encoding the viral genome of CHV1-Euro7 show greater potential to biologically control chestnut blight than their cytoplasmicly-infected counterparts. Three treatments were employed to compare transgenic hypovirulent (TG), cytoplasmic hypovirulent (CH), and virulent (V) strains. To produce ascospore inoculum, naturally occurring and artificially established punch-initiated cankers were spermatized by painting cankers (PI) three times each summer (2004-2010) with a conidial mixture containing MAT-1 and MAT-2 mating types of the appropriate treatment strain (TG, CH, or V). Conidial inoculum was produced by inoculating scratch wounds (SI) made to the bark with a mycelial slurry of the appropriate treatment strain (TG, CH, or V). Non-treated trap (T) trees were left to monitor natural canker development as well as hypovirus spread. Tree condition and natural canker establishment were assessed for all trees in August of each year. As of August 2010, there were 181 natural cankers in TG plots, 107 in CH plots, and 107 in V plots. Cankers were sampled, when detected and each November, to determine the hypovirus infection status of the thallus. Although the purpose of the spermatization treatment was to produce ascospores, many treated cankers also acquired hypovirus from the treatment inoculum and have begun to produce callus.

Some significant findings this past year include the increased detection of hypoviruses, both in the canker thallus and perithecial outcrosses. Beginning in 2007 and continuing through 2010, hypoviruses have been detected in cankers on trap trees, both in the thallus and ascospores. Transgenic inoculum has been detected not only in TG plots but also in CH and V plots. Ascospore colonies were assessed by pigmentation and morphology. To date, 84,441 individual ascospores from 3,548 perithecia have been examined.

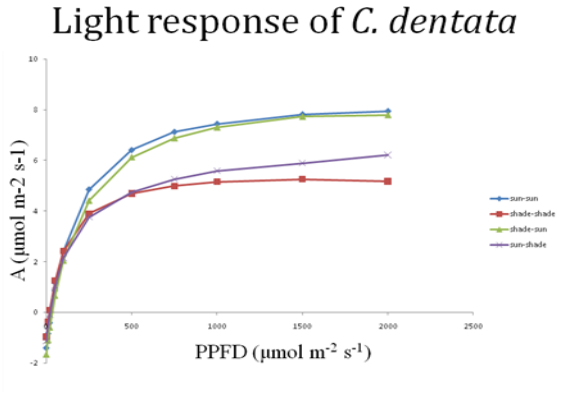
Tree mortality, as of July 2010, was greatest in the virulent plots (85%), followed by TG plots (76%) and CH plots (67%).

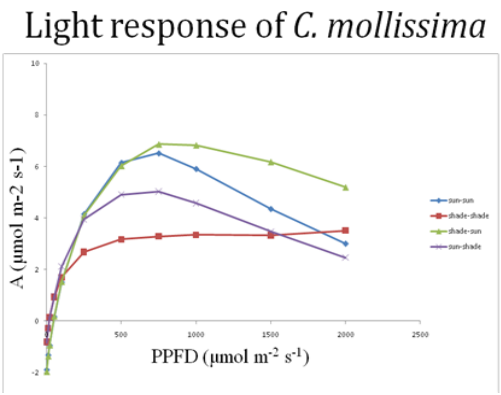
OBJECTIVE 3. To investigate chestnut reestablishment in orchard and forest settings with special consideration of the current and historical knowledge of the species and its interaction with other pests and pathogens.

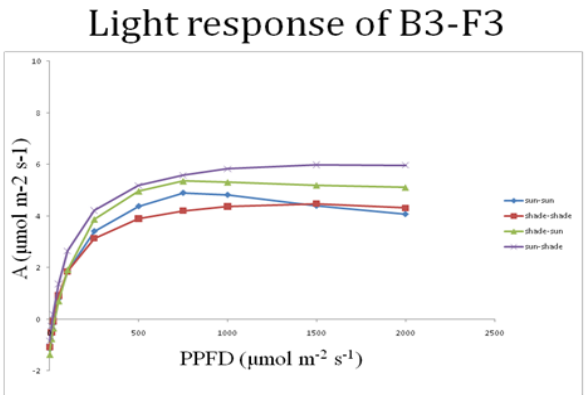
Keaton Hughes, University of Tennessee-Chattanooga

Determining the shade-tolerance of B_3-F_3 chestnut hybrids. Hughes stated that *Castanea mollissima* differs from *C. dentata* in many ways. In comparison, *C. mollissima:* does not persist in the shade; it has a shorter and wider stature; and, it is blight resistant. No one is looking at the physiology of B_3F_3s, so the question is: Are the backcross trees more similar, physiologically, to American, European or Chinese chestnut? To measure the physiology of trees, they utilized a Li-Cor 6400 gas-exchange analyzer. It uses a controlled environment and the environment can be manipulated in terms of photosynthetically active radiation, CO_2 concentrations and relative humidity. Also gases (particularly CO_2) are measure and compared to a known control sample. By measuring the gases before a stimulus is applied to a leaf, they can determine the photosynthetic outputs of each leaf.

Hughes define terms: LCP = light compensation point, a point at which carbon losses due to respiration are offset by carbon gains via photosynthesis; Amax = maximum assimilation of CO_2(maximum amount of photosynthate produced by a leaf); and LSP=light saturation point or the point where an increase in light no longer produces an increase in photosynthate. Shade tolerant plants generally have a low LCP, LSP and low Amax. Shade intolerant plants have a higher LCP and LSP and a relatively high Amax. Chinese chestnut has been classified as shade intolerant and American chestnut is shade tolerant. In March 2010 with funding from TACF®, Hughes planted 48 individual American, Chinese and B_3F_3 trees in 3-gallon pots. One-half of each group were grown in complete sunlight while the other half were grown under 50% shade cloth to simulate growing under a forest canopy. The plants were moved outdoors in June and half of each treatment was switched with regard to it light treatment (i.e. 12 plants that were grown in full sunlight were switched to shade) in order to determine the relative plasticity of the plants. The plants were rotated within each group to eliminate any microclimate effects. Li-Cor measurements take about 30 minutes each.







Keaton's conclusions were:

- *A*max values of B_3F_3 hybrids are similar to those of *C. mollissima,* however:
- Hybrids do not appear to suffer photoinhibition (as *C. mollissima* does)
- Hybrids exhibit LCP values more comparable to *C. dentata* than *C. mollissima,* suggesting a similar shade-tolerance

Scott Schlarbaum, University of Tennessee, TN Agricultural Experiment Station

East Tennessee State Nursery Planting. A small planting, about 100 trees was planted in conjunction with the American Chestnut Cooperator's Foundation at the East TN nursery. There is good growth and survival. The material is all one genetic family.

Mammoth Cave National Park. This planting contains multiple ACCF and TACF® planting (full-sib American chestnuts). These plantings have poor survival—loss to due to *Phytophthora* sp.

Pennsylvania Bureau of Forestry plantings. This planting contains pure American chestnut seeds from TACF® that were raised in the Georgia nursery and transplanted to PA in 2001 . The seedlings were of very high quality and data is taken annually. Tree shelters were initially used due to the high deer pressure in PA. The area is now fenced. The trees are now sizeable, but some do have blight and mortality has occurred.

Restoration project using backcross B_3F_3 trees. The goal of this project is to provide chestnut restoration prescriptions to land managers working in forests within the species' natural range. The objectives are:

- To test breeding generation and chestnut size on nursery seedling morphology
- To test field performance of chestnuts planted in silviculturally treated forest stands.

The process of testing the B_3F_3 generation is:

- Fall 2007—USDA Forest Service, Southern Region secured material from TACF®; material transferred to Dr. Stacy Clark (USDA Forest Service, Southern Research Station); Dr. Clark is the lead scientist for this project; UT Tree Improvement Program cooperates with her and the Southern Region.
- Winter 2007/08—sowed nuts at GA state nursery and established nursery study
- Summer 2008—UT conducted nursery photosynthesis study and monitored development in nursery.
- Summer 2008—developed site prescription and selected three stands for planting
- Summer 2008—tested sites for *Phytophthora cinnamomi* in soil
- Fall and Winter 2008—prepared sties for planting
- Winter 2008—developed experimental design for planting; sowed nuts for 2010 plantings at TN state nursery
- February 2009—lifted and processed seedlings for planting using experimental design
- February 2009—established plantings at three National Forests in the southern region
- May and September 2009—collected bud break data and survival, light and competition data
- Winter 2009-10—collected seedling growth and survival data; sowed nuts for 2011 plantings; readied sites for 2010 planting
- March 2010—established two plantings on two National Forests in southern region.
- May and September 2010—collected bud break data and survival, light, and competition data
- May –August 2010—selected six sites for 2011 plantings in Region 8

  2009 plantings:
  - 1497 chestnuts:
    - American: 318, 3 families
    - Chinese: 192, 1 family
    - B_1F_3: 280, 2 families
    - B_2F_3: 260, 2 families

- B_3F_3: 426, 5 families

2010 plantings:

– 1356 chestnuts:
  - American: 278, 3 families
  - Chinese: 120, 1 family
  - B_1F_3: 127, 2 families
  - B_2F_3, 195, 2 families
  - B_3F_2, 142, 2 families
  - B_3F_3, 499, 7 families

2011 Plantings

– 1907 seedlings
  - American: 325, 1 family
  - Chinese: 233, 1 family
  - B_3F_2, 151, 1 families
  - B_3F_3, 1198, 10 families

BC_3F_3 Nursery Study 2008 and 2009

- Grown as 1-0 bare-root seedlings to maximize growth potential (Kormanik et al. 2004)
- Sowed nuts by breeding generation and chestnut size group (Large vs. Small) (2 replications). Height difference is related to nut size.
- Measured height, root-collar diameter, and number of first-order lateral roots
  Results:
  - Large chestnuts had larger height and root collar diameter growth, compared to small chestnuts, but did not differ in number of roots
  - Generations differed in seedling growth (Chinese>B_1F_3>B_2F_3>B_3F_3>American)
  - No family differences

Schlarbaum noticed that oak seedlings have episodic growth, i.e., flush/stop/flush/stop. Chestnuts on the other hand, tend to grow more continuously and not until August do they put on a lot of height growth and girth.

How big are the seedlings that are being planted?

- 2009 - 3.1 ft height (0.6-6.3'), 0.5 inch root-collar diameter (0.08-1.3") = modified KBC bar planting
- 2010 – 4.4 ft height (0.3 to 8.6'), 0.6 inch root-collar diameter (0.07 to 1.2") = Shovel/auger planting
- Grown to get above competition and deer pressure

Schlarbaum stated that seedlings that are much over 5' after one growing season are difficult to lift and there is lot more transplant shock.

2009 and 2010 B_3F_3 Field Tests

Site prescription:

- Shelterwood with reserve (residual BA=10-20 ft^2/acre)
- Site index >70 ft (base age 50-NRO)
- Stumps sprouts of undesirable competitors treated with herbicide

The current status of budbreak from the 2009 plantings is: Chinese (a) > B_2F_3 (ab) > B_1F_3 (b) > B_3F_3 (c) > American (d).

The current status of the 2009 planting in NC is:

- Survival is 90%
- Dieback on 32%
- Deer browse on 9%
- 85% of the trees are free-to-grow
- Red maple (14%) and yellow popular (13%) were the most abundant understory competitors

The current status of the 2009 planting in TN is:

- Survival is 94%
- Dieback on 94% (most related to deer browse)
- Deer browse on 84%
- 96% of the trees are free-to-grow
- Striped maple (26%), black cherry (13%) and black locust (13%) were the most abundant understory competitors
- Mesh shelters were erected in spring 2010

The current status of the 2009 planting in VA is:

- Survival is 94%
- Dieback on 65% (most related to deer browse)
- Deer browse on 42%
- 95% of the trees are free-to-grow
- Yellow poplar (23%) and sassafras (16%) were the most abundant understory competitors
- Mesh shelters were erected in spring 2010

2010 Plantings

- Two sites were planted n VA and TN
- Similar experimental design and data collection protocols as 2009 plantings
- Trees were tall at plangent (avg 4.4' up to 8.6')
- Budbreak data show same trends as 2009 plantings

2011 Plantings

- Six planting sites selected in VA, NC and TN National Forests
- Will test effects of forest management practices on chestnut survival and growth
- Shelterwood harvest
- Midstory removal treatment (first stage of shelterwood harvest)
- Replicated three times

The midstory treatment involves removal of midstory using a hack and squire herbicide injection to increase light to the forest floor by 20-25%. An experimental planting will be utilized and the overstory will be removed in 4-6 years.

Schlarbaum indicated that outplanting is expensive. By the time that salaries, supplies, equipment and trailers are included, outplanting expenses are high.

Leila Pinchot, University of Tennessee, TN Agricultural Experiment Station

Studying American chestnut reintroduction. Pinchot's main project is a forest planting in the Daniel Boone National Forest in southeastern KY. Chestnuts were planted into an existing forested area. She planted in low (95% canopy cover), medium (88% canopy cover) and high (40% canopy

cover) light levels. She planed 750 bare root chestnuts that were grown at the Georgia State Nursery. The seedlings were lifted and measured in February 2010 and planted in March 2010. Pincot planted 300 American chestnut (from 5 families), 300 B_2F_3 (SA330 and SA417) and 150 Chinese chestnut from the Forest-Keeling Nursery. The variables that she measured included: height; root collar diameter; available PAR; canopy cover; stomatal conductance; chlorophyll levels in the leaves; and, timing of budbreak. The objective is it to see how the different chestnuts grow in the different silivcultural treatments. Unfortunately, the trees suffered a high level of mortality (American 39%; B_2F_3 34% and Chinese 5%). She has problems with ink disease, *Phytophthora cinnamomi*. What is interesting is that the Daniel Boone NF is not an area where she would expect to find *Phytophthora*. It is not a low land area and it is not wet. Her initial thought was that it was brought it from the nursery. Pinchot took soil samples from areas near where the chestnuts were planted and Steve Jeffers at Clemson verified that the soils tested positive for *Phytophthora*. Another problem that may have contributed to the mortality was insect predation, caused by the chestnut sawfly, *Craesus castanteae*. The leaves were completely defoliated to the petiole. The trees reflushed only to be defoliated again. It is potentially problematic in the replanting area. Predation was much higher in the treatments with lower light levels. She hypothesized that the higher light level treatment allowed the leaves to produce more photsynthate and secondary metabolites. Also, there was more predation on American and backcross trees than on Chinese chestnut. She noticed that almost all of the seedlings are highly chlorotic. She is collecting leaves and soil to test for nutrient content. (Anagnostakis suggested manganese deficiency).

Yale-Myers forest planting. Pinchot described the planting she did in Connecticut using Anagnostakis' material. Four-hundred, thirty-two family B_2F_1 (from Chinese) and B_3F_1 (from Japanese), planted at the Georgia State Nursery and outplanted in April 2009 into shelterwood thinning with gaps. By September 2010, there was 63% mortality, mostly from deer browse.

Spatial Nursery Study. Nearly 2,000 seedlings were planted (the 2011 crop for Stacy Clark) with the goal of looking at height growth patterns with regard to distance between adjacent seedlings. Pinchot took data on Chinese (314 seedlings); American (238 seedlings); B_3F_2 (314 seedlings); and, B_3F_3 (989 seedlings) seedlings. Height measurements were taken weekly for the first half of the summer; measurements are now every two weeks. Data is still being collected.

Pam Kazmierczak, UC Davis

Chestnut cultivars. Cultivars have been in the field for three seasons. Despite alkaline conditions, the trees have put on 2-3 feet of new growth. Two requests have been filled for propagation material—one from a nursery in Washington State for dormant, graftable material and a second from a California nursery for shoot tips to be used for *in vitro* propagation. The requests were for all eight cultivars in the collection. These include: *Marrone Comballe; Marrone di Chusa Pesio; Marrone do Marradi; De Coppi; Eaton; Campbell NC-8; Luvall's Monster*; and, *Quing*.

Sandra Anaganostakis, Connecticut Agricultural Experiment Station

Gall wasp. Anagnostakis is conducting a gall wasp experiment in conjunction with Stacy Clark (USDS-Knoxville) and Henry McNab (USDA-Bent Creek, NC) to test if gall wasp resistance is heritable, as chinquapins are resistant to gall wasp. The crosses were made in 1993 and the trees were planted at Bent Creek in 1995 in an area that had both gall wasp and chestnut blight. All of the controls died (pure species). Data is as follows:

Female parent	Male Parent	# Trees Planted	# Survivors in 2009
American 1	Ozark chinquapin 1 x Chinese 1	6	1
American 2	Ozark chinquapin 1 x Chinese 1	18	1
American 3	Ozark chinquapin 1 x Chinese 1	20	5
	Male Parent 1		
American 4	Ozark chinquapin 2 x Chinese 2	49	29

Similarly, Anagnostakis showed data comparing 2006 and 2009 with regard to cankers and galls on the surviving trees. It was apparent that one male parent clearly did not have sufficient blight resistance for them to survive (M1). The other male parent survival was much better (M2). The data are as follows:

Male	Blight Cankers on Trunks*				Galls on Terminal Branches**					
	2006		2009		2006			2009		
	+	-	+	-	0	1-10	>11	0	1-10	>11
M1	17	0	7	0	6	7	2	6	1	0
M2	20	16	28	1	5	18	13	25	3	1

*Chestnut blight disease cankers present (+) or absent (-) on trunks.
**Gall wasp numbers: 0=no galls detected on branch terminals; 1-10 galls noted on branch terminals; and, 11 or more galls noted on branch terminals.

Resistance to gall wasp appears to be heritable and probably is not cytoplasmic because the female parents were American chestnuts. More directed crosses are needed. The crosses have been done and Anagnostakis is hoping to put out the chestnuts in Ohio where gall wasp is present.

Crosses made. A total of 556 chestnuts were harvested from hand-pollinated crosses in the fall of 2009 and 527 open pollinated nuts were collected. These include orchard and timber selections for planting at CAES and by collaborators. Open-pollinated seed were collected from *Castanea henryi* (Chinese chinquapin) and some of its hybrids at the chestnut planting at Sleeping Giant in the fall of 2009 for continuing studies of resistance to Asian chestnut gall wasp, in collaboration with Stacy Clark, USFS and private orchard owners in Ohio where the wasp is now a serious threat to the nut crop.

Seedlings from hand-pollinations between individual Ozark chinquapin trees at Lockwood Farm are being raised in the greenhouse for planting at Lockwood Farm in 2012 and testing for resistance to chestnut blight. A cooperative project with the Cherokee Nation in Oklahoma will allow for planting of some of the trees on Cherokee land to determine survival potential.

Selected chestnut cultivars were grafted in the greenhouse in the spring of 2010 and were planted at Lockwood Farm to increase the cultivar collection. More grafts will be made in late September to compare grafting success with those in the spring.

Maintenance of timber chestnut selections at Windsor and Griswold continues and collections of seed in Windsor will be sent to Forest Keeling Nursery (Missouri) to produce seedlings for the Fairfield Garden Club's 2012 Centennial Project of planting 100 backcrossed timber chestnut trees on

public land in Fairfield County. Future seedlings from Windsor and Griswold will be used for planting in areas in Connecticut and other northeastern forests where timber harvests have released native chestnut sprouts.

Weather issues. Anagnostakis showed a graph of precipitation in CT in 2010 and while there was a large amount of rain in March, there has been very little rain since. It also has been very warm in CT in 2010—nearly three weeks ahead in growing degree days. She just finished planting and it was time to pollinate; she is having trouble keeping up.

Flora Patterson, mycologist (1847-1928). Anagnostakis portrayed Flora Patterson at the 100th year Plant Science field day at the Experiment Station. Patterson was the first woman hired by the USDA. In 1904, Patterson received American chestnut bark samples from the NY Botanical Garden and identified the canker disease as a fungal pathogen.

Erin Coughin, Berry College

Coughlin is an undergraduate student at Berry College in Rome, GA and she is the Georgia-TACF® intern, working under the direction of Dr. Martin Cipollini.

Phytophthora control in Georgia nurseries. *P. cinnamomi* has been an issue in a few specific locations in Georgia, namely the Berry College backcross orchard and at the University of Georgia Horticulture Research Center backcross orchard. At the Berry College orchard on Lavender Mountain, *P. cinnamomi* was not expected to be an issue because the orchard location is in a mountainous area. Nevertheless, starting about a year after the chestnuts were planted, mortality due to root rot began. It was confirmed, through root and soil tests, that *P. cinnamomi* was responsible for killing a significant number of trees. At the UGA Horticulture Center in Watkinsville, it is suspected, although not confirmed, that *Phytophthora* was present. This Coastal Plains region is warm, moist and contains poorly drained agricultural soils, an ideal location for *Phytophthora*.

To treat *P. cinnamomi* at both locations, Georgia-TACF® has been using a treatment recommended by Steve Barilovits (Carolinas-TACF®). A combination of Prudent 44 (marketed as a liquid fertilizer) and Nutrol (marketed as a fungicide) were sprayed onto the leaves at the rate of Prudent 44 (1 ounce/gallon) and Nutrol (2 ounces/gallon) using a backpack sprayer. Georgia-TACF® treats trees once per month during the growing season, although minimum treatment frequency has not been determined. Both products can be ordered through Lidochem.

The treatment was highly successful. In the Berry College orchard, *Phytophthora* problems were nearly eliminated. No seedlings have died from verifiable *P. cinnamomi* since the treatment began. The orchard is thriving; 3-year old trees are now 15' tall. At the UGA orchard, no trees have died from verifiable *Phytophthora*. Coughlin experienced no leaf burn, something Georgia-TACF® was concerned about at the beginning of the experiment. In conclusion, a combination of Prudent 44 and Nutrol are effective as preventative and therapeutic treatments for *Phytophthora* in Georgia nurseries.

Lynne Rieske-Kinney, University of Kentucky

Asian chestnut gall wasp update. *Dryocosmus kuriphilus*, is a potentially devastating pest of chestnut that causes round or globular twig, shoot, and leaf galls on actively growing shoots. Galls act as vegetative sinks that influence the nutrient and defense status of developing tissues. Galling reduces tree vigor, prevents normal shoot development and can cause tree mortality. Galling also prevents infested shoots from producing new shoot growth and reproductive flowers, thereby reducing or eliminating nut production. Chestnut production and chestnut restoration efforts throughout the eastern USA are threatened by the persistent spread of this exotic, invasive insect. The Asian chestnut gall wasp was first observed in the USA in 1974 infesting Chinese chestnut near Bryon, GA, and has since spread north to Pennsylvania and Maryland, and westward to central Kentucky. Asian gall wasp attacks all chestnut species. Rieske-Kinney's research program has been evaluating the mechanisms associated with gall formation and characterizing associates of the Asian chestnut gall wasp in eastern North America, to more fully understand gall development and what factors may regulate gall wasp populations.

The gall wasp lays its eggs into dormant buds that will form the following spring. The galls are in a dormant state all winter and it is not until budbreak occurs that infestation is evident. There is rapid formation of globular galls; each contains one-to-several chambers. Each chamber within the gall will contain developing gall wasp larvae. A single gall can produce several wasps. The gall provides food and habitat for the gall-inducer and they alter the plant physiologically with quantitative and qualitative changes in signaling compounds. On a whole plant level, the galling prevents normal shoot development, causes shoot and branch dieback, reduces flowering, nut production and in extreme cases, tree mortality.

Gall wasp has expanded north-northeasterly from Byron, GA. The hot spots are now in northern Ohio and she believes she will be working soon in Pennsylvania. Her research objectives are to:

- Characterize the natural enemy complex associated with ACGW. This includes recruitment of the enemies, identification of those enemies, identifying the mechanisms by which they cause gall wasp mortality and any evolving interactions that may occur.
- Develop a means of rearing or manipulating gall wasp populations under controlled conditions.
- Evaluate the spatial distribution of ACGW, including geographic distribution, within-orchard distribution, and within-tree distribution.
- Assess the effects of orchard floor manipulations to optimize habitats for natural enemies of ACGW.

With regard to objective 1, she has collected galls from the leading edge of ACGW geographic range, with the help of Sara Fitzsimmons and Gary Micsky. Shorty after ACGW was introduced into North America, biological control agents were introduced at the point of the ACGW introduction. The records for this are very poor; there were some species of *Torymus* and another genus of parasitic wasp. These were released at the hot spot of the introduction in 1976. Rieske-Kinney is interested if the parasites followed the gall wasp as the gall wasp expands its geographic range. She learned early on that parasitized galls were quite common. She spent some time looking at this more closely and found that when parasite populations are high, gall wasp populations are low and conversely, when parasite populations are low, gall wasp populations are high. Thus, parasites are a major source of gallmaker mortality. She posed two questions: (1) what are the parasites? and, (2) how do they work?

Looking at gall wasp parasites in general, the female parasite oviposits directly into the gall and the eggs of the parasite hatch and then feed directly on the host insect. This can occur as an entirely internal process, indicating a highly evolved relationship between the host and the parasite. In contrast, there can be an external process, whereby the eggs of the parasite are laid in close proximity to the host insect and the parasite larvae still feed on the host, but it is a much looser relationship.

There are six parasitic insects: one introduced Torymus species and five native species (oak-associated). The most numerous species is *Torymus sinensis*. The second-most common insect is *Ormyrus labotus*, a common parasitoid. Results from gall dissections are:

- Parasitoid species indistinguishable as larvae
- Impossible to determine which species are affecting ACGW populations
- Focused on two numerically important species

She and student, Rodney Cooper, looked at emergence from collected galls of the two parasites (data from VA, KY and OH). She reported that all of the *T. sinensis* are found in the older galls not the current galls. With regard to *O. labotus*, it is found in both older and newer galls, with the exception of the KY site.

Parasitoid prevalence

Site	*T. sinensis*	*O. labotus*	Setting
VA	.55	.28	Plantation/orchard
KY	0	.41	Forest
OH	.71	.04	Plantation/orchard

The hypothesis was that the earlier a site was infested with gall wasp, the greater the parasitoid load would be. This is not the case, as the OH site was the most recently infested among the three sites. The settings are different as the KY is a forest mosaic setting. Perhaps parasitoid recruitment is more dependent on the surrounding vegetation than it is on the time infestation became established.

Are the two parasitoids impacting ACGW similarly and are there any type of evolving interactions between the two? Since the larvae are indistinguishable, R. Cooper developed a molecular approach to determine which larvae were present in which gall population. He developed a PCR-amplified ITS2 sequence which confirmed presence of:

Dryocosmus kuriphilus 600 bp
Torymus sinensis 520 bp
Orymus labotus 560 bp

In the native forest site (KY), *O. labotus* is having more impact than *T. sinensis*. In contrast at Meadowview, *T. sinensis* is the more prevalent of the two parasitoid species.

Species	Summer	Fall	Winter	Spring
D. kuriphilus	Eggs hatch in dormant bud	Larvae dormant	Larvae dormant	Larvae feed; galls expand
T. sinensis	Larvae feed on gall wasp	Pupa	In dormant galls	Lay eggs in gall; larvae feed on gall wasp
O. labotus	Has multiple generations			

Dryocosmus kuriphilus and *T. sinensis* both have one generation per year. The first generation of *O. labotus* feeds on the gall wasp. The second generation has no ACGW to feed on (either killed by parasites or fully developed), so this generation instead attacks *T. sinensis* which is what remains in the gall. Subsequent generations attack oak galls. The first generation is perfectly synchronized with ACGW and the second generation is well synchronized with *T. sinensis*. There appears to be an antagonistic relationship evolving between the introduced parasitoid (*T. sinensis*) and the native parasitoid (*O. labotus*), both of which are using the ACGW as the host. *T. sinensis* appears to be moving with expanding gall wasp populations as it was found in OH quickly after the gall wasp was detected. Because *O. labotus* is historically a oak parasite, it is found in association with oak gallmakers in forest settings. Conclusions to this objective are: (1*) T. sinensis* is expanding with the gall wasp; (2) native natural enemies that appear to be finding the gall wasp as it expands its geographic range; and (3) there are interesting relationship between the native and the introduce parasitoid.

Objective 2 is to develop a means of rearing or manipulating gall wasp populations under controlled conditions. She is working with obligate parasites of the host plant, so a viable host plant is necessary. From experience, she knows that the gall wasp does invade, attach and develop on seedling chestnuts. She has tried many times to artificially infest seedlings and she has failed. She hit on the idea this year of doing this through grafting. She collected scion wood from galled chestnut and grafted onto potted seedling stock. Rieske-Kinney went to Chattanooga to tap the expertise of Hill Craddock who spent time showing her how to graft. The grafts were completed in TN and were taken back to Lexington. She was thrilled to find her grafts had galled; 18% of the grafts generated gall wasps that successfully developed through adulthood. This provides a tremendous opportunity; there are all sorts of aspects of the gall wasp biology and ecology that now be answered because the gall wasp populations can be manipulated. She now has Ignazio Grasioso, a new student, who will use grafting to answer some fundamental questions about gall wasps. She also hopes to manipulate the orchard floor to see what kind of effect it might have because complex environment is likely to provide refugia for natural enemies, as well as pollinators, that might impact the gall wasp.

Amelia Harris, University of Tennessee at Chattanooga

A census of naturally occurring American chestnut and a floral survey at Bendabout Farm, Bradley County, TN. Harris sees a need for an American chestnut census at Bendabout Farm. She hopes to provide a description of the site (location; geology and soil; climate; and past and present land use). Her goals are to locate naturally occurring chestnuts and describe the flora at the farm. For the chestnut census, she hopes to take data on:

- Specific habitat
- Sunlight density
- Disturbance level
- Best areas to begin reforestation of TACF® hybrids

Floral survey data will include:

- Create a complete biological inventory
- Better document plant species at Bendabout
- Locate any species of concern

Bendabout Farms is in Bradley County, TN and it comprised 4,000 acres that encompasses several different habitats. There are several small chestnuts growing in the forest, presumably from rootstock. The highest elevation at the farm is 315 m and the lower is 233 m. One reason for selecting Bendabout Farm is that there is not much known about chestnut in this ridge/valley area of TN. Average rainfall is 138 cm and the average temp is 15.8 C. The growing season is between 180-220 days. There is some interesting land use at Bendabout Farm. The land was deeded to the Johnson family in 1841 and they lived harmoniously with the native Cherokee. The farm is famous for its polo matches. The pines on the farm (2,300 acres) are systematically burned; they were planted most for quail hunting.
Bendabout Farm has been working with TACF® since 1993 when Bill Raoul established the first TN research orchard on the property. The property was planted with transplant from GA, TN, MS. They were kept alive with hypovirulent strains, but they began to succumb to *Phytophthora* in 1997. In 1996, a second orchard was planted using TACF® B_2F_2 and in 1997, a third orchard was planted using seed from the first orchard.
Her two objectives are:

- Locate naturally occurring surviving chestnut
    - Better document the occurrence of American chestnut in the ridge and valley region
    - Select best possible reforestation site at Bendabout Farm
- Generate a list of vascular plants on the Farm and document species of concern

Harris began her study in June 2010 and she has made 11 plant-collecting trips to Bendabout Farm. Her preliminary results are: 161 species have been identified (2 of which are relatively uncommon—spider lilies); 18 introduced species. She also has found one *Castanea dentata* and one *Castanea pumila*. Based on other data, she can estimate that she will find 500-600 species during the course of her study. This fall she hopes to learn the best survey methods for chestnut census. She will continue to make weekly trips to Bendabout until it is too cold and in the winter months she will identify grasses and sedges that she has collected.

Tom Saielli, University of Vermont

Preliminary research to restore American chestnut to northern hardwood forest. His focus is on the cold tolerance of American chestnut. His focus is on:

- Identification of seed sources exhibiting greater cold hardiness; and,
- How different seed sources and canopy cover might influence growth and winter injury

Saielli posed the question, “why study cold tolerance?” In the northern limits of the American chestnut, it was found mostly at low elevations along rivers and near large bodies of water. It was suggested that American chestnut was prone to freezing. Saielli commented that pure American chestnut and backcross breeding lines do suffer winter injury, exhibiting terminal shoot dieback. American chestnut is less cold tolerant than Red oak or Sugar maple, not surprising since their range is much farther north. This is important since most of the breeding stock comes from the mid-Atlantic and southern states. Will these materials be adaptive to New England? We need information on the cold tolerance of Chinese chestnut. So far, it is believed that Chinese chestnut is significantly less cold tolerant than American chestnut. He wants to use this information to bolster cold tolerance in the TACF® breeding program. There is only one breeding orchard in Vermont and none in New Hampshire. He discussed three points:

- Reproductive material is often most limited in cold hardiness.

- Are American chestnut nuts cold hardy enough to survive in northern winters and allow for natural reproduction?
- How do chestnuts compare to nuts of naturally reproducing trees in the northeast?

He obtained 1,120 American chestnuts from different states including northern (NY, ME, VT, NH), central (NJ, MD, PA) and southern (KY, VA, NC) regions along with four Red oak (VT, NH) and Chinese chestnut sources (CT, VT, PA, MD).

In the laboratory, he sampled five nuts from each of the twenty-four sources and exposed them at temperatures from 4°C to -4°C and monitored them for cellular breakdown. When breakdown was noticed, that was considered level at which cold tolerance threshold had been reached. These data were used to do statistical analysis to determine if there are significant differences between species and sources. Red oak (RO) and American chestnut (AC) were not significantly different and both were significantly different from Chinese chestnut. The Chinese chestnut (CC) had less cold tolerance (-6°C) than RO and AC (-12°C). With regard to regional differences, AC from the south were not as cold tolerant as the central and northern sources. Environmental sources, elevation and source are all important components. One cannot assume that nuts from a northern state are automatically more cold tolerant. Some of his most cold tolerant sources were from ME, VT and also PA. Nuts from Catskill, NY were not as cold tolerant even though they are from a northern state.

He studied the growth and winter injury of seedlings in a northern hardwood forest (Green Mountain, VT). He tested 13 pure AC, two CC and four RO sources in a replicate design under three different canopy types (closed, partially closed and open). The seedlings (n=806) were started in the greenhouse in the spring and the sites were prepared installing deer cages and vole shelters. He did treatment verification by taking a picture of the canopy at each seedling and he measure temperature variations. He used a SLR camera with a hemi-spherical lens and imported the photos into Gap Light Analyzer 2.0, which converted the pictures into a black/white image. The white pixels were analyzed and a formula was used to calculate canopy percent. Statistics indicate that there are significant differences among his treatments. On average, the percent openness in his treatments was 56% for the open plots, 20% for the partially closed canopy and 18% for the closed canopy. He had temperature probes (in the plots (20 cm and 120cm above ground) and the air temperature analysis was that open plots were colder (-26°C) than the partial and closed canopy plots (-23°C). In terms of growth, Saielli measured:

- Height of shoot in cm
- Diameter at base in mm
- Winter injury after bud break in the spring

Statistical analyses of above data were done on the canopy treatments and among the three species and he looked for source difference among the 13 sources of American chestnut. The open treatment was taller (significantly higher than partially closed and closed canopy) and the open treatment also had a greater diameter. Consequently, open canopy had more winter injury. In terms of species height, Chinese chestnut was taller than American which was taller than Red oak. Chinese chestnut had more winter injury than either American chestnut or Red oak.

In terms of the variability of American chestnut sources, there were significant differences among the sources, but not among the regions—there was no pattern in terms of region, as seen in the following data:

Am. Chestnut Source	Total Height	Winter Injury
Castile, NY	A	A
Haun, PA	AB	A
Atkins, VA	AB	AB
Bell Hollow, VA	AB	AB
Lasdon, NY	BC	AB
Tindal, NJ	CD	AB
Cochester, VT	CD	AB
Somerset, MD	CD	AB
Michaux, PA	DE	AB
Rockport, ME	DEF	B
Metecalf, KY	EF	B
Wagon Road, NC	FG	B
Atkinson, ME	G	B

Source is important as are elevation, region, environmental resources (nutrients, etc.) and hydrology. Trees that grow taller seem to experience more winter injury.

His conclusions were:

- There was significant nut and shoot cold tolerance variation among species.
- Variation among American chestnuts (and maybe Chinese) sources matters.
- Growth and winter injury were significantly influenced by silvicultural treatment.
- Trade-offs between growth and winter injury (the more open the canopy, the more growth-but those open canopies were colder and there was a lot more winter injury).

Gary Micsky, Penn State

2010 Research/Demonstration Orchards. PA plantings in 2010 were as follows:

Farm and Location	Freeman Tree Farm, Knox, PA	Sumner Tree Farm, Pulaski, PA	Burnham Tree Farm, Washington Co., PA	Lawango Run Farm, Greenville, PA
Date Planted	4-27-2010	4-29-2010	4-3-2010	4-29/30-2010
Volunteers	4	6	14	2
Volunteer Hours	34	30	28	12
Seed	30 OP w/tube/cage 30 F1 w/tube/cage 50 OP Random	50 OP (Haun) 30 F_1 (Haun)	12 OP (Haun) 12 F_1 (Haun)	90 F_1 (TACF®)

Identifying Potential Sites/Growers for Outplantings. Participants at March 24, 26, 31 "Grower Schools" were given an opportunity to take home 10 open pollinated seed in exchange for agreeing to provide baseline follow up data regarding their success or failure in growing chestnut seedlings on their site. Over 1,000 open pollinated seed were distributed to 102 individuals with follow-up surveys scheduled to begin in September 2010. Surveys will be used to determine: (1) grower dedication; and, (2) site suitability for future outplantings. Baseline data will include: % seed surviving; height of

seedlings; weed and pest controls; tree protection; and, problems encountered as of September, 2010. Three new test/demonstration orchards including open pollinated and F_1 seedlings were established near Pulaski, Knox, and Greenville PA. One demonstration orchard established in Washington County PA.

Locating and Collection of Local Genetic Materials.

- Controlled pollination to produce F_1 seed on three American chestnut trees in Haun Orchard, Sandy Lake, PA.
- Balloon pollination of Beagle tree, Mercer, PA (07.09.2010) with American chestnut pollen. Four new surviving American trees over 6-inch dbh identified near Grove City, PA

Outreach Efforts. "Chestnut Chatter" an extension mailing list developed in 2008 and adapted to a Penn State listserv in 2009, accommodates the need to quickly notify 51 trained volunteers of program activities such as: pollination schedules; harvest dates; and, other labor intensive activities.

"Chestnut Gall Wasp – Monitoring a New Threat" a Penn State Cooperative Extension fact sheet, was developed with assistance from Lynne Rieske-Kinney, University of Kentucky an NE-1033 participant. Fact sheet was utilized in to enlist volunteers in monitoring the spread and severity of this pest in western PA and eastern OH. This fact sheet was published in Penn State Cooperative Extension newsletter *"The Woodlander"* (Summer 2010) and featured in the Penn State Ornamental Growers' IPM Update (Northwest PA Edition) Summer 2010 Issue #3.

Carmen Medina-Mora, Michigan State University

Pollination studies of American chestnut in Michigan. Michigan orchards are a mixed population of cultivars and seedlings. She hopes to determine which combination of cultivars grown in Michigan orchards will result in maximum pollination and nut production. Her rationale is: maximum yield potential will only be realized when pollination is 100%. She is also interested in the health of nuts and their nutritional value. There are many Euro-Japanese hybrids grown in Michigan because of the large nut size. This cultivar has problems in that it is male sterile, so pollinators are needed. Chestnuts require cross-pollination and she provided a schematic of female flowers. Many Michigan orchards have challenges. She posed a number of questions:

- Is pollen/flower timing critical?
- What cultivars pollinate early or late?
- How synchronous are the pollinizers with flower receptivity?
- Can Chinese cultivars efficiently pollinate European cultivars in an orchard? And vice versa?
- Does incompatibility affect fruit set at pollination, fertilization or during the development of the embryo?
- How much pollen is needed?
- When is pollen needed?

Her first objective was to answer what is the appropriate time for pollen and flower receptivity in relation to the outcome, nut set and production. She conducted some controlled pollination studies (in 2008, 2009 and 2010) using 'Colossal' as the main female. The pollen sources were 'Okei' and 'Benton Harbor' located at distant chestnut plots from the experimental plots. The application of pollen was once, twice or three times on the same flowers. In order to do this work, she had to define

certain stages: pre-anthesis; anthesis; and, post-anthesis. In terms of male pollen her data are as follows:

Catkin Type	Pollen Length	Cultivar
Astaminate	No Pollen	'Colossal'
Brachystaminate	1-3 mm	
Mesostaminate	3-5 mm	'Benton Harbor'
Longistaminate	5-7 mm	'Okei'

She tested viability and germination of 'Benton Harbor' and 'Okei' pollen and they are comparable, so the slight morphological difference did not inhibit the production of pollen.Her data included flower efficiency (number of flowers hand-pollinated versus number of burs harvested) and nut set (how many fully developed nuts were harvested). The highest nut set was pollinating the flowers three times during the summer. Her pollen application dates were: June 26 (early); July 3 (mid); and, 9 July (late). She also looked at nut quality—sinkers (healthy) versus floaters (poor quality), along with nut weight of single nuts, location of nuts within the bur. She chose another orchard in 2009 and used four dates (23 June, 2 July, 7 July and 4 August). For those flowers pollinated four times, she collected 34 good nuts of the 75 harvested (that was the highest percentage of her treatments). Harvest dates 2 (2 July) and 3 (7 July) produced good quality nuts. Single pollination event of 4 August was too late (no nut production). Time 2 and 3 were the crucial times for pollination.

In terms of natural pollination, Medina-Mora chose an orchard where she monitored pollen shedding and flower receptivity. She utilized 1 pollinizer tree ('Okei') to five 'Colossal' trees. The 'bagged flower date' represented a flower which was exposed to pollen until a particular date. Conversely, other flowers were bagged and exposed to pollen at other dates. Data were as follows:

Number of nuts naturally pollinated by 'Okei' during 2008

Exposed Until:	Flowers	Burs Harvested	Nuts
18 June	60	1	0
26 June	60	2	0
3 July	60	23	4
11 July	60	35	17
23 July	60	58	20

Data from 2009 were as follows:

Exposed Until:	Flowers	Burs Harvested	Nuts
24 June	148	100	8
30 June	148	125	45
6 July	148	131	68
3Aug	148	69	45

Based on these data, she concluded that the peak time for pollination, based on hand-pollination and natural pollination studies in Michigan, is from late June to early July.

The following table represent flowers in which the bags removed on certain dates.

Bags Removed:	Flowers	Burs Harvested	Nuts
26 June	120	109	20
30 June	120	118	28
6 July	120	62	4
7 Aug	120	85	3
28 Aug	120	24	0

2009 was a poor year for nut production. This led to speculation that the general cool, wet summer reduced crop load. The 2009 data confirmed this as natural pollination produced fewer nuts than hand-pollination, indicating the flowers were still fertile when pollen was supplied.

Medina-Mora concluded by stating that orchards need more than one pollinizer (early, mid and late). The longer flowers are exposed to pollen, the greater the chance that three nuts will be produced. Pollination is an additive effect. She also defined pollinator and pollinizer. A pollinator is an agent (animal or insect that contacts a flower), and a pollinizer is the source of pollen.

Dennis Fulbright, Michigan State University

Full service chestnut state. Michigan is now #1 in: (1) number of chestnut farms in the U.S.; (2) amount of acreage; and (3) more hypovirulence than any other state. California leads the nation in yield. Fulbright attended a chestnut conference in Italy in October 2009, and it was the first European chestnut conference and the fifth Italian chestnut conference. On a farm field trip, they went to a chestnut cooperative near Cuneo (Associazione Produttori Castagna Val Pellicle), which modernized only eight years ago. The Italian cooperative has 80 members; Michigan has 40. With the ancient trees in an ancient culture, it might be assumed that their chestnut yield is high. The Italian cooperative and the Michigan cooperative both formed in 2001. Their yield in 2001 was 14,000 pounds the first year. They began with large trees. The peak year was 2007 when they harvested about 50,000 pounds. However, in 2008, their production was only 8,000 pounds. The reason for the decline was a lot of rain (2 weeks) during the pollination period. This was the first time Fulbright had ever heard of pollination issues in Europe. Michigan chestnut growers had to begin with small trees. They began with 2,000 pounds in 2001. Yield increased to 50,000 pounds in 2007. Since then, yield has declined due to severe frost in 2008-10.

'Labor Day' is a cultivar that is a Korean chestnut growing in Michigan. It has good yield, early production. It is probably blight resistant. 'Benton Harbor' is a Chinese chestnut cultivar that has the best growth of any tree in the Michigan orchards but it breaks in the wind. The buds, however, grow back, produce shoots and produce nuts. It is a good pollinizer for 'Colossal'. It is a large, robust tree with good yield. 'Everfresh' is a Chinese chestnut cultivar with a 2-year shelf life. It has good yield and produces good size nuts. It is blight resistant. The nuts have far less mycotoxins than 'Colossal'.

The Midwest Chestnut Farm Conference was held 21-22 August 2010 with the intent to prepare for a SCRI grant, set priorities, mix researchers with growers, and establish an extension component.

Business Meeting

While there was no official business meeting (Sandra Anagnostakis is the 2011 chair and the meeting will be held at the Incarnation Center, Ivoryton, CT, October 27-30, 2011), Bill MacDonald spent time conferring with the group about the 2012 International Chestnut Meeting. The meeting will be held at the National Conservation Training Center, 698 Conservation Way, Shepherdstown, West Virginia 25443-4024 (http://training.fws.gov). The first item of discussion related to when to host the meeting. The general consensus was to hold the meeting the week of Labor Day, September 2012 (Wednesday Sept. 5 through Sunday, Sept. 9). Fulbright indicated that the China meeting used concurrent sessions and he suggested strongly that we avoid concurrent sessions. Side trips were suggested, possibly as spouse tours:

- Antietam Battlefield
- National Arboretum
- USDA Lab at Bardane
- Harper's Ferry (1/2 day) or Georgetown (1/2 day)
- Washington, DC (whole day)
- Shepherdstown
- Chestnut Tour Field Trip—orchards in area (Sugarloaf?)
- Maryland Vineyards (there was a vineyard tour in China)

Suggested Oral Sessions included:

- Marketing/Production
- Orchard Management
- Propagation/Grafting/Rootstock
- Genetics/Diversity/Databases
- Breeding
- Pests/Pathogens
- Cryphonectria and Hypoviruses
- Food Science
- Transgenics
- Forest Ecology/Reforestration

There was discussion about a publication. While ISHS produces a publication, it may be up to the organizers to edit the publications.

It was suggested to have one email address where people can get information. Meg Staton agreed to use the *Fagaceae* website as a portal for information. There were pros and cons for the TACF® website. Dana Nelson suggested the University Conference Center could handle inquires and registration. There was a suggestion that all sessions should be filmed for internet programming; others felt that this might violate the ISHS copyright rules.

With regard to a keynote dignitary, Hill Craddock and Brad Hillman suggested former President Jimmy Carter. Craddock volunteered to call the President to check on his availability.

Respectfully submitted,
Mark Double
West Virginia University

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Completion of proposed milestones:

2005:

- Characterization of the role of hypovirus p29 in virus RNA accumulation in *C. parasitica*, and virus transmission through conidia of the fungus:

Completed early:

Suzuki, N., Maruyama, K., Moriyama, M. and Nuss, D. L. Hypovirus papain-like protease p29 functions *in trans* to enhance viral double-stranded RNA accumulation and vertical transmission. *J. Virol.* **77**:11697-11707, 2003.

- Generation of polyclonal antibodies against 5 overlapping regions of hypovirus ORF B, and construction of a *C. parasitica* EST database.

Both completed in 2004.

2006:

- Publication of a *C. parasitica* EST database containing approximately 2500 ESTs.

Completed early.

Dawe, A.L., McMains, V.C., Panglao, M., Kasahara, S., Chen, B. and Nuss, D.L. An ordered collection of expressed sequences from *Cryphonectria parasitica* and evidence of genomic microsynteny with *Neurospora crassa* and *Magnaporte grisea*. *Microbiology* **149**:2373-2384, 2003.

2007:

- ORF B polyprotein processing pathway in *C. parasitica* confirmed, ORF B mature proteins responsible for altering fungal cell signaling pathways mapped and DNA microarray analysis of hypovirus-mediated alteration of fungal gene expression initiated.

ORF B polyprotein processing pathway not completed. Microarray analysis initiated giving publication in 2003.

Allen, T.D., Dawe, A.L. and Nuss, D.L. Use of cDNA microarrays to monitor transcriptional responses of the chestnut blight fungus *Cryphonectria parasitica* to infection by virulence-attenuating hypovirus. *Eukaryotic Cell* **2**:1253-1265, 2003.

2008:

- Polyprotein processing maps completed for hypoviruses CHV1-EP713 and CHV1-Euro 7, and a detailed view compiled of the changes in cellular transcriptional profiles caused by infection of C. parasitica with mild and severe hypoviruses.

  Polyprotein processing map has not been competed. Transcriptional profiles caused by mild and severe hypoviruse have been generated. The *C. parasitica* EST microarrays have also been used to examine the effect of hypovirus infection on G-protein signaling and to expose a linkage between mitochondrial and viral hypovirulence.

Allen, T.D. and Nuss, D.L. Specific and common alterations in host gene transcript accumulation following infection of the chestnut blight fungus by mild and severe hypoviruses. *J. Virol.* **78**:4145-4155, 2004.

Dawe, A.L., Segers, G.C., Allen, T.D., McMains, V.C. and Nuss, D.L. Microarray analysis of *Cryphonectria parasitica* Gα- and G$\beta\gamma$- signaling pathways reveals extensive modulation by hypovirus infection. *Microbiology* **150**:4033-4043, 2004.

Allen, T.D. and Nuss, D.L. Linkage between mitochondrial hypovirulence and viral hypovirulence in the chestnut blight fungus revealed by cDNA microarray analysis. *Eukaryotic Cell* **3**:1227-1232, 2004.

2009

- Determine specific genetic fingerprints of tested cultivars through the use of microsatellite

markers and find loci useful for parentage analysis (Fulbright)

- Refine the genetic linkage and genome sequence maps for map-based cloning of fungal vic and pathogenicity genes
- Tom Kubisiak used the *C. parasitica* Genome sequence generated by the JGI Community Sequencing Program to identify 689 simple sequence repeats (SSRs) and designed 141 primer pairs. One hundred and thirty Four of the 141 primer pairs amplified discrete products, with 96 of the 134 showing polymorphism for the JA17 and X17.8 parents of the mapping cross. Allele data were generated for 96 progeny from the mapping cross for 32 polymorphic SSRs and a total of 30 of these markers were placed within the context of the published *C. parasitica* linkage map (Kubisiak and Milgroom, 2006, FG&B 43:453-463). The new linkage data were used by the JGI finishing group to connect a number of scaffolds in Version 1 of the genome assembly to generate Version 2 that was released July 10, 2009.
- Complete assembly and community manual annotation of the *C. parasitica* genome sequence Nine-hundred and ninety-five of 11,251 transcripts have been manually curated by the *C. parasitica* annotation team to date. Version 2 of the genome assembly has been released by the JGI consisting of 26 scaffolds. Five of the new scaffolds contain two teleomers and are of a length consistent with a complete chromosome. Six additional scaffolds contain one teleomer and are in excess of 1 MB in length. The assembly release Version 2 of whole genome shotgun reads was constructed with the Arachne assembler and improved with finishing reads. This release contains 26 main genome scaffolds totaling 43.9j Mb. Five scaffolds are considered complete teleomere on one end. The remaining 15 scaffolds are smaller and do not contain teleomers. Roughly half of the genome is contained in four scaffolds all at least 5.1 Mbp in length. Annotation of Version 2 assembly was produced by the JGI Annotation Pipeline using a variety of homology-based and ab initio predictors. The Version 1 Gene Catalog and its manual curations also were mapped to the Version 2 assembly and were included in the filtering procedure that determined the initial Version 2 Gene Catalog. After filtering for EST support, completeness and homology support, a total of 11, 609 genes were structurally and functionally annotated.
- Use the *C. parasitica* genome sequence to develop new microarray chip and proteomics platforms for analysis of global gene expression in the blight fungus when challenged by viral pathogens
- Orchard established in WV with advanced, back-cross chestnut trees from VA for assessment of host resistance with hypovirulence in the *Cryphonectria parasitica* population—replanted in 2009.

In progress.

2010

- Complete characterization of *C. parasitica* antiviral RNA silencing pathways.
  Two Dicer genes were identified in the *C. parasitica* genome, cloned and disrupted. Dicer DCL2 was shown to be required for antiviral RNA silencing while Dicer DCL1 was not required (Segers et al., PNAS 2007, 104:12902-12906). Four Argonaute genes were identified in the *C. parasitica* genome, cloned and disrupted. Only Argonaute AGL2 was required for antiviral RNA silencing (Sun et al., PNAS, IN PRESS). Three RNA dependent RNA polymerases and an orthologue of the QIP exonuclease involved in transgene silencing in *Neurospora crassa* have been identified in the *C. parasitica* genome and are currently being cloned and disrupted. The C. parasitica gene *oah*, encoding the enzyme Oxaloacetate acetylhyrdolase (OAH), a member of the PEP mutase (PEPM)/isocitrate lysase (ICL) superfamily, that catalyzes the hydrolysis of oxaloacetate to oxalic acid and acetate, was cloned, characterized and disrupted. Knockout of the *oah* gene reduced the ability to form cankers on chestnut trees, which suggest that the enzyme plays a key role in virulence.

Completion of milestones not proposed:

- A proposal to sequence the *C. parasitica* genome was approved by the Department of Energy Community Sequencing Program in June of 2006.
  The assembled 8.5 *X C. parasitica* genome sequence was released to the public on September 30, 2008. This is a tremendous resource for future studies on the chestnut blight fungus and its interaction with the chestnut tree.
- Demonstration that hypovirus p29 suppresses RNA silencing in *C. parasitica* and in heterologous plant system. This is the first report of a mycovirus-encoded suppressor of RNA silencing.

Segers, G.C., van Wezel, R., Zhang, X., Hong, Y. and Nuss, D.L. Hypovirus Papain-like protease p29 suppresses RNA silencing in the natural fungal host and in a heterologous plant system. *Eukaryotic Cell* **5**:896-904, 2006.

- Demonstrated that RNA silencing serves as an antiviral defense mechanism in *C. parasitica* (first example for any fungus) against hypoviruses and mycoreoviruses.

Segers, G.C., Zhang, X., Deng, F., Sun, Q. and Nuss, D.L. Evidence that RNA silencing functions as an antiviral defense mechanism in fungi. *PNAS USA* **104**:12902-12906, 2007.

- First report of the cloning and sequence analysis of mycovirus-derived small RNAs (vsRNAs) generated by RNA silencing. The vsRNAs were shown to be produced, in a dicer *dcl-2*-dependent manner, from both positive and negative hypovirus RNA strands at a ratio of 3:2 and to be non-randomly distributed along the viral genome. *C. parastiica* was shown to respond to mycovirus infection with a 10-15 fold increase in *dcl-2* transcript acuumulation while the expression of *dcl-1* was modestly increased. The expression of *dcl-2* was further increased (~35 fold) following infection by a CHV1-EP713 mutant that lacks the p29 suppressor of RNA silencing. A similar response in dicer gene expression following virus infection of plants or animals has not yet been reported. In this regard, it is anticipated that the evolutionary position of fungi relative to animals and plants will provide insights into additional novel

mechanisms for the induction and suppression of RNA silencing pathways yet to be revealed in the other organisms.

Zhang, X., Segers, G.C., Sun, Q., Deng, F. and Nuss, D.L. Characterization of hypovirus-derived small RNAs generated in the chestnut blight fungus by an inducible DCL-2-dependnet pathway. *Journal of Virology* **82**:2613-2619, 2008.

- Virus RNA recombination is an important component of virus evolution that contributes to the emergence of new viruses and the generation of internally deleted mutant RNAs, termed defective interfering (DI) RNAs, that are derived from, and dependent on, the parental viral genomic RNA. We provided the first experimental evidence that a host RNA silencing pathway is required for DI RNA production and virus vector RNA instability for a single-strand, positive sense RNA virus.

Zhang, X. and Nuss, D.L. A host dicer is required for defective viral RNA production and recombinant virus vector instability for a positive sense RNA virus. *Proc. Natl. Acad. Sci. USA.* Early Edition, October 13, 2008.

Participants in NE-1033 Meeting
Silverbell Lodge, Cataloochee Ranch, Maggie Valley, NC
September 17-18, 2010

Front row (seated) from left: Eric Fang, Dana Nelson, Paul Sisco, Fred Hebard, Dennis Fulbright
Second row: Fenny Dane, Jeanne Romero-Severson, Leila Pinchot, Ignazio Graziosi, Sandra Anagnostakis, Carmen Medina, Bill MacDonald, Don Nuss
Third row: William White, David Bevins, Bode Olukolu, Pam Kazmierczak, Debbie Wilk, Erin Coughlin, Angus Dawe, Shin Kasahara, Ken Jensen
Fourth row: Ron Sederoff, Kathleen Baier, Tom Kubisiak, Sara Fitzsimmons, Kendra Gurney, John Dougherty
Fifth row: Amelia Harris, Katie D'Amico, Bill Powell, Lynne Rieske-Kinney, Ali Barakat, Tom Saielli, Josh Springer
Back row: Bert Abbott, Ana Metaxas, Dragan Galic, Andy Jarosz, Russell Regnery, Andy Newhouse, Scott Schlarbaum, Jimmy Maddox, Brad Hillman, Xuemin Zhang, Hill Craddock
Not pictured: John Carlson, Gil Choi, Mark Double (photographer), Keaton Hughes, Gary Micsky, Mariah Sappington, Diane Shi, Meg Staton