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

Incarnation Center, Ivoryton, CT

October 28-29, 2011

Attendance:

Connecticut:	Sandra Anagnostakis—Chair, Pamela Sletten (Connecticut Agricultural Experiment Station)
Indiana:	Jeanne Romero-Severson (University of Notre Dame)
Kentucky:	Lynne Rieske-Kinney (University of Kentucky)
Maryland:	Donald Nuss, Gil Choi, Ken Jensen (University of Maryland Institute of Bioscience and Biotechnology Research, Shady Grove)
Michigan:	Andrew Jarosz, Dennis Fulbright, Claire Moore, Josh Springer (Michigan State University)
Mississippi:	Dana Nelson (USDA-FS, Southern Institute of Forest Genetics)
Missouri:	Mark Coggeshall (University of Missouri)
New Jersey:	Bradley Hillman—Administrative Advisor (Rutgers University)
New Mexico:	Angus Dawe (New Mexico State University)
New York:	Steven Jakobi (Alfred State College), Lilibeth Northern (SUNY-ESF)
North Carolina:	Paul Sisco, (TACF®, Asheville)
Pennsylvania:	John Carlson (Penn State University), Sara Fitzsimmons, (TACF®, State College), Mike Marshall (Shippensburg University)
Tennessee:	Hill Craddock, Adam Lyon (UT Chattanooga), William White (UT Chattanooga, TACF®)
Vermont:	Kendra Gurney (TACF®, Burlington), Paul Schaberg (USDA-FS, South Burlington)
Virginia:	Fred Hebard, Laura Georgi, David Bevins, Eric Coalson (TACF®, Meadowview)
West Virginia:	William MacDonald, Mark Double, Eric Goddard (West Virginia University)
Japan:	Shin Kasahara, Takuya Takahashi (Tohoku University, Sendai)
Switzerland:	Sarah Bryner (Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Birmensdorf)

The meeting was called to order by Chairman Anagnostakis at 9:30 am on October 28, 2011 at the Incarnation Center, Ivoryton, CT.

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

Jeanne Romero-Severson, University of Notre Dame

EST ssr markers (in conjunction with Mark Coggeshall, University of Missouri). All of the cultivars in the Horticulture Agroforestry Research Center (HARC) collection are being genotyped to make sure the correct name is applied. The Chinese chestnut repository was started by Ken Hunt in 1996 with 2 trees of each of 69 cultivars. The 2001 cultivar trial was started with 5 trees each of 12 cultivars—planted on 27' centers. Coggeshall collected leaves and twigs from all repository trees and cultivars and sent them to Romero-Severson. There are a number of different kinds of DNA markers that can be used to characterize natural populations. In the last 5-6 years, a particularly useful marker type has been available—expressed sequence tags (ESTs). Sometimes embedded in those sequences are simple sequence repeats (ssr) or microsatellites. It turns out that these sequences make exceptionally good markers for studies that involve multiple members of the same species (*C. dentata, C. mollissima, C. crenata*, etc). Unlike genomic microsatellites, these work well across the genus. These were the types of markers that were used. Because they are expressed, they can blast the sequence and go to NCBI, where millions of sequences are stored, and get an idea of what genes these sequences are embedded in. That does not mean that they can make functional associations with a set this small, but it is useful information. They only tested 30 sequences from the *Fagaceae* genomics database and a third of those were useful.

Assessment of cultivar identity and relatedness using SSR-containing EST sequences from *C. mollissima:* Putative identities of seven Chinese chestnut contigs

- cc54_contig12496_v2
  - match to gi|262512524|*Quercus robur*
- cc454_contig2033_v2
  - similar to KNAP2_MALDO Homeobox protein knotted-1-like 2
- cc454_contig9659_v2
  - similar to putative lipid phosphate phosphatase 3, chloroplast precursor
- cc454_contig13496_v2
  - similar to Pleckstrin homology domain-containing protein 1
- cc454_contig350_v2
  - similar to syntaxin-like protein
- cc454_contig353_v2
  - similar to WRKY transcription factor
- cc454_contig3815_v2
  - similar to nucleobase-ascorbate transporter

Criteria

- One "band" of expected size across 10 different Chinese chestnuts

- 64C annealing temp
- Contig match to plant EST database (NCBI) at e^{-20} or better.

What do they get when they use these markers—an estimate of the size of the fragment. They are not doing sequencing, just estimate of fragment size. The purpose of the study was to see if these ESTs are polymorphic in this set of cultivars. It turns out that they are polymorphic.

Different names, same genotype

Cultivar	M12496		M2033		M9659		M13496		M350		M353		M3815	
Armstrong	127	136	469	469	273	273	343	343	272	295	221	236	179	185
Armstrong	127	136	469	469	273	273	343	343	272	295	221	236	179	185
Crane	127	133	469	469	278	278	343	343	284	286	230	230	176	179
Orrin	127	133	469	469	278	278	343	343	284	286	230	230	176	179
Orrin	127	133	469	469	278	278	343	343	284	286	230	230	176	179
Orrin	127	133	469	469	278	278	343	343	284	286	230	230	176	179
Auburn Super	124	127	469	469	267	277	343	350	290	298	236	236	176	185
Av-Super	124	127	469	469	267	277	343	350	290	298	236	236	176	185
Jersey Gem	124	127	469	469	267	277	343	350	290	298	236	236	176	185
Peach	124	127	469	469	267	277	343	350	290	298	236	236	176	185
Super	124	127	469	469	267	277	343	350	290	298	236	236	176	185

In the above tables, if two cultivars are the same, the genotypes (fragment sizes) are expected to be identical. However, in the tables below, cultivars of the same name do not have identical genotypes.

Same name, different genotypes

Cultivar	M12496		M2033		M9659		M13496		M350		M353		M3815	
Kohr	112	130	469	469	269	273	343	350	286	290	236	236	179	179
Kohr	112	130	469	469	269	273	343	350	286	290	236	236	179	179
Kohr	111	129	469	469	268	273	343	350	285	289	236	236	179	179
Kohr	112	133	469	469	267	269	340	340	278	291	226	232	173	176
Kohr	124	133	469	469	266	269	343	343	278	299	230	230	176	179
Miller_72-76	127	130	469	469	273	273	343	343	294	294	230	233	182	182
Miller_72-76	127	130	469	469	273	273	343	343	294	294	230	233	182	182
P316_149	127	130	469	469	273	273	343	343	294	294	230	233	182	182
Miller_72-76	130	130	469	469	269	269	343	343	286	299	221	236	179	179
Miller_72-76	124	124	469	469	273	278	343	343	299	299	230	230	179	179

Their size estimates are accurate to within a base pair. One cannot assume that all the above 'Kohr' cultivars are identical genotypically.

A total of 104 repository trees, representing 45 different cultivars were genotyped using 7 EST-SSR markers in 2009. The results suggest that 19 trees (~18%) were mislabeled. An additional 6 trees need to be re-done. A total of 94 trees in the cultivar trial, representing 26 different cultivars were genotyped using 7 EST-SSR markers in 2009. Results suggest that 10 trees (~10%) were mislabeled. An additional 11 trees need to be re-done.

The summary is as follows:

- Microsatellite markers from EST contigs show moderate to high polymorphism in *C. mollissima*
- Four of seven markers showed high sequence conservation with either *Q. robur* or Q. *petraea*
- FGP genomics tools can facilitate interdisciplinary collaborations involving both applied and basic science

Approach to NSF to include *Castanea* collection in the *Fagales* (in conjunction with University of Missouri and the Missouri Botanical Garden). Coggeshall and Romero-Severson submitted, in mid-October 2011, a grant to NSF who put out a call for their 'living collections initiative'. In addition to chestnut, they also have at HARC: *Juglans, Carya, Quercus* and *Corylus* sp. These living collections include 738 unique accessions, representing 31 species and 19 interspecific hybrids within the order *Fagales*, as show in the following table:

Genus	Species	Hybrids	Accessions
Alnus	2	4	10
Betula	3	1	7
Carpinus	2	0	10
Carya	3	1	138
Castanea	5	8	78
Corylus	2	1	15
Juglans	6	2	259
Pterocarya	1	0	1
Quercus	8	2	220
Total	31	19	738

The living collections of tree and shrub species established at HARC provide opportunities to investigate the genetic basis for: annual variations in phenology, growth and nut yields; adaptation to climate change; and, tolerance to important pests.

The proposal is designed to support efforts to enhance, secure and curate this collection for the research community. This proposal submission represents collaboration between: University of Missouri; University of Notre Dame; University of Missouri Dunn-Palmer Herbarium; and, the Missouri Botanical Garden in St. Louis. The research Plan includes:

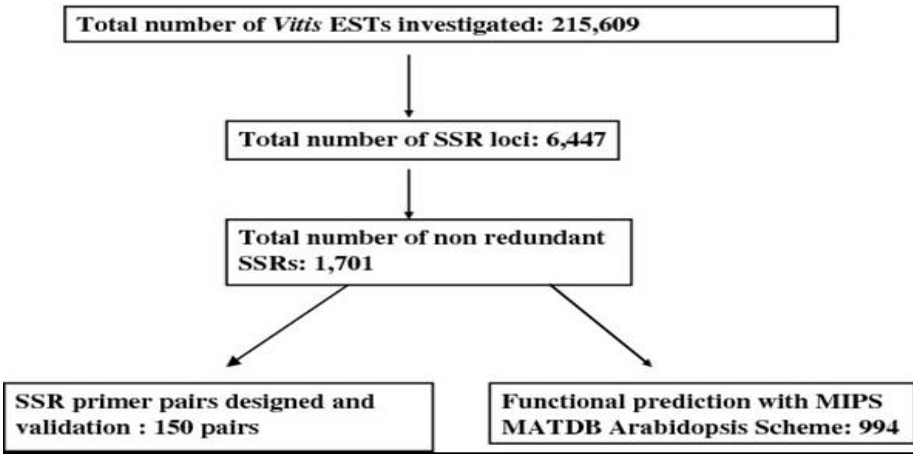
- web portal development (MU)
- collection of plant voucher specimens (MU, UMO, MOBOT)
- link web-based voucher images to new NSF sponsored "cloud-based" web platform
- DNA barcoding of each accession using both nuclear and chloroplast genome EST-SSRs (ND)
- hosting of DNA sample archives and barcode sequences (ND, MU)

The University of Missouri Dunn-Palmer Herbarium, founded in 1896, has:

- 170K total accessions
- 95K accessions available on TROPICOS® (Tropicos® is an electronic database containing 1.2 million scientific names, 3.9 million specimens and 169K digital images).
- Voucher sheets (including fruits) will be prepared by UMO herbarium staff and deposited in both the UMO and MOBOT herbaria.

The proposed plan is to use EST SSRs from the nuclear genome and use actual sequence, using large chunks of the chloroplast genome. To give NSF an example, Coggeshall and Romero-Severson, provided the following protocol from *Vitis*.

DNA barcoding of each accession using both nuclear and chloroplast genome EST-SSRs



Nuclear genome barcodes developed from FGP and EST sequences for European oaks

Goal: Develop >20 EST-SSR regions (400 bp min.) per tree

Chloroplast barcodes developed from large single copy region of the *Q. rubra* chloroplast (~80% of oak chloroplast genome)

While the *Vitis* protocol calls for 150 pairs (a very high standard), the *Castanea* goal is 20.

EST-SSR workflow. Illustrating EST-SSR successfully validated or associated with functional predictions relative to available EST sequences for the genus *Vitis* (Huang et al. 2011).

Genetic characterization of *Castanea*. The great thing about genetic fingerprinting when using markers that can be used across genera, is that those same markers can be used for multiple purposes. When supporting a nut industry, the following are important:

Genetic fingerprinting

- Cultivar ID
- Cultivar pedigree
- Cultivar relationships
- Germplasm characterization
- Populations dynamics in natural stands
- Preservation and conservation of species
- Postglacial migrations
- Evolutionary history

Nut production

Restoration

If restoring a species to the forest, then there is some overlap with nut production. As a grower, you need an assurance of identity. Growers must have confidence in cultivar characteristics. Growers want efficient, meaningful genetic and phenotypic evaluations across locations, growers and academic programs, and you do not have that if you do not know the genetics of the material. In order to patent a cultivar, there needs to be rock solid barcodes.

If the cultivar pedigree is known:

- Performance of progeny to parents and grandparents can be compared
- Quantitative traits can be compared (nut size or nuts/kg)
- Gene action (recessive, dominant) if Mendelian can be assessed
- Efficiency of breeding for stress resistance can be increased
- Quantitative performance can be improved

Cultivar relationships

- Related cultivars may have similar performance
- Relatedness among parents may affect progeny performance (in two ways: If you have two good parents that work well together [called 'combining ability' in the corn industry], they can produce great things. At the opposite end of the spectrum, two parents that are not compatible may not fertilize one another or the seeds may be poor quality).

What makes a good marriage?

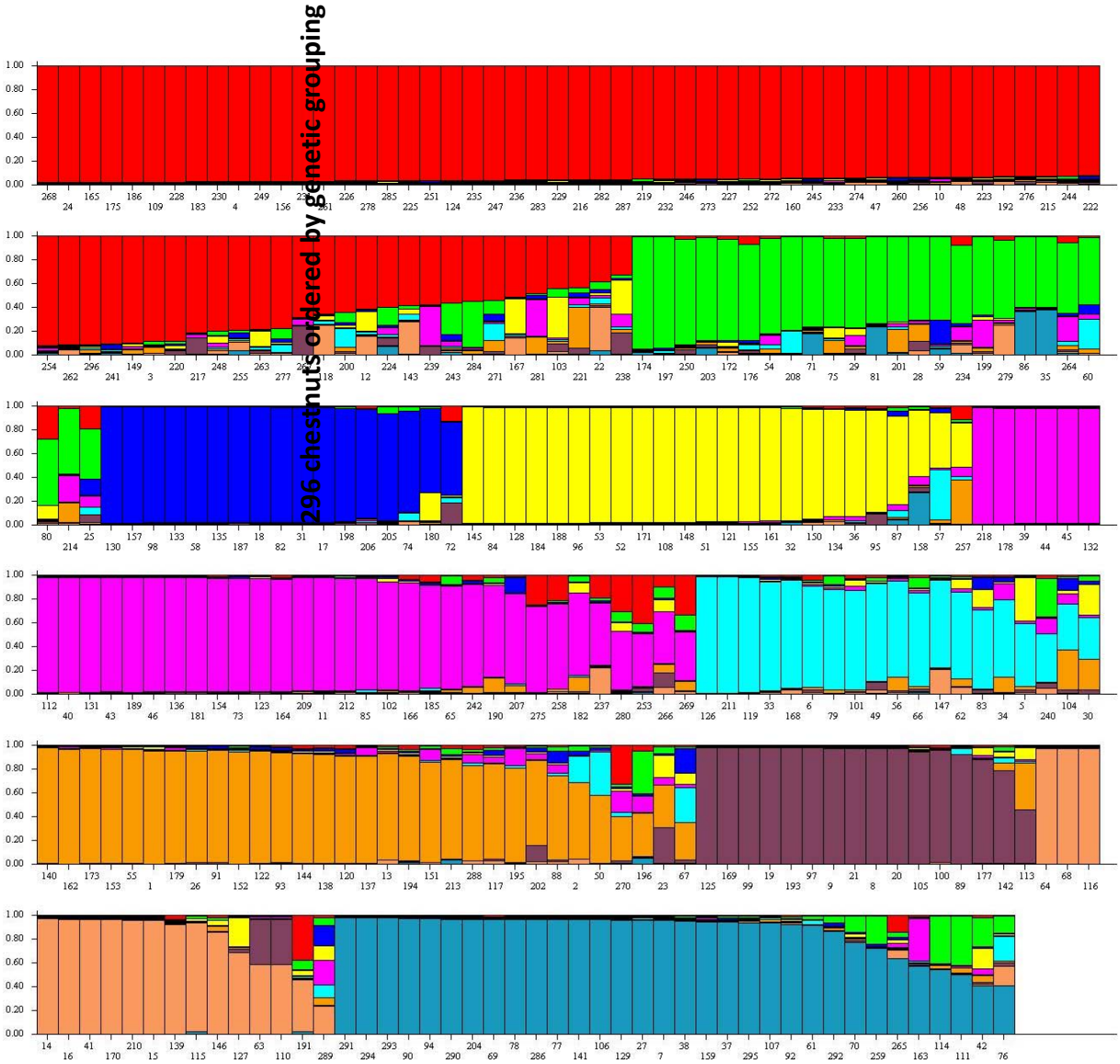
- Genetic compatibility
    - Good seed set, sound seed, vigorous seedling, good juvenile growth, stress tolerant, good parent…
- Incompatible genetics
    - Poor seed set or infertility
    - Endosperm breakdown/kernel collapse
- Poor genetic compatibility
    - Slow seedling growth
    - Failure to thrive
    - General susceptibility
    - Reproductive issue

There are chestnut complications:

- Many species, many "hybrids"
    - European, Chinese, Japanese, American….
- Years of cultivar development for nuts
- Inadequate germplasm source characterization (i.e. 'Colossal' is a J X E but what European?)
- Lost or never recorded pedigrees.
- Restoration success ≠ cultivar success

Genetic tools and data for chestnut

- EST-SSR markers
- A set of DNA sequences from (mostly) real genes
    - 48335 contigs
    - 3226 ready to test microsatellite containing sequences
    - NSF-PGRP 0605135 .
    - "Genomic Tool Development for the Fagaceae"
- Genotypes for 296 chestnut cultivars
- 10 major genetic groups



The same color in the above chart (of 296 cultivars) does not mean that the genotypes are the same; it means that they are genetically similar. This is a first good look at genetic diversity among cultivars.

The strategic plan is as follows:

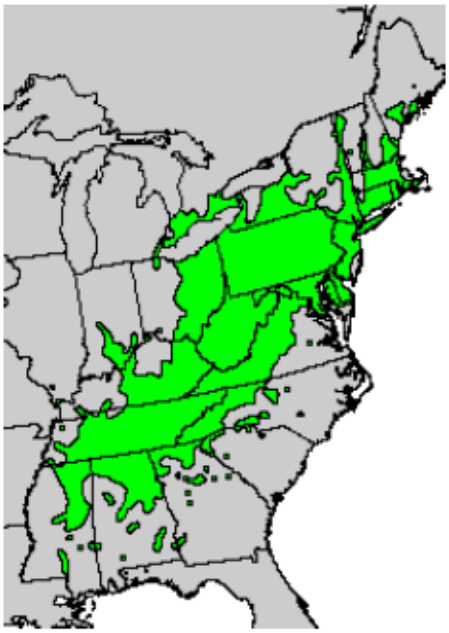
- Develop a bombproof species-specific detection kit for the chloroplast
- Develop DNA markers for cultivar identification
  - Amplifiable, robust and informative in *C. dentata, C. mollisima, C. crenata, C. ozarkensis, C. sativa*
  - Sequence amplicons
- Establish genetic databases for the source species
- Identify species ancestry for cultivars
  - If one species, which one
  - If hybrid, which species are ancestors
  - If hybrid, degree of "hybridity"
- Pry loose funding from the NSF or USDA to conduct range-wide population differentiation study in *C. dentata*

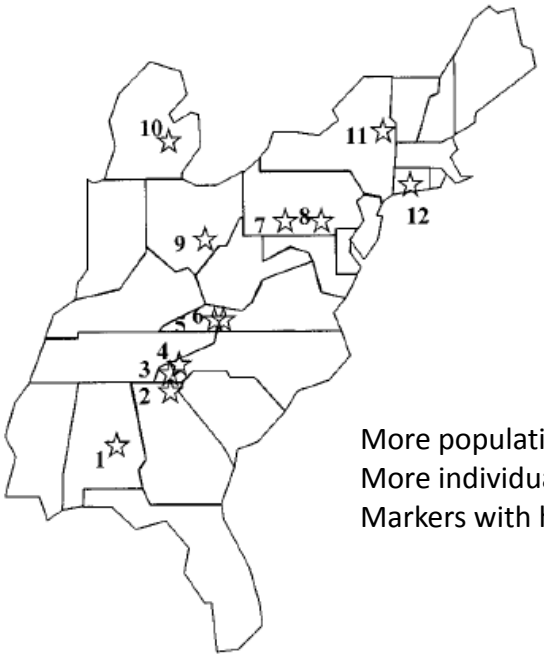
Strategy:

- Collect 30-60 unrelated individuals from each species.
- Select a test panel of six individuals from each species.
- Produce amplicons from the 10 regions of the chloroplast that show polymorphism in the Fagaceae.
- Sequence amplicons.
- Distinguish intraspecific from interspecific polymorphism.
- Verify species specificity.
- Genotype cultivars.
- Genotype natural populations of *C. dentata, C. ozarkensis.*

In an F_1 interspecific hybrid, the chlorotype reveals the species of the seed parent. If a "pure" species individual has a chlorotype of a different species, there was an interspecific cross once upon a time.

With the current set of markers, Romero-Severson would like to do a range-wide differentiation in genetic diversity study in existing stands of American chestnut. In 2011, the markers are so much better and so much cheaper than when Tom Kubisiak et al. did a genetic study of chestnut years ago. To conduct a good study, 30-35 chestnut individuals over some sort of defined area (less than a county in size) are needed. What they need now are more samples from more sites.





More populations
More individuals/site
Markers with higher PIC

Allozyme and RAPD analysis of the genetic diversity and geographic variation in wild populations of the American chestnut (Fagaceae). Hongwen Huang, Fenny Dane, Tom L. Kubisiak, American Journal of Botany 85(7): 1013–1021. 1998.

What is possible in 3-4 years:

- Collect enough cultivars/populations to approximate a fair representation of the major species.
- Develop a bombproof species-specific detection kit for the chloroplast
- Develop a robust set of nuclear markers and do:

  - Species assignment
  - Hybrid detection and classification
  - Cultivar definition and identification
  - Genetic diversity and differentiation for *C. dentata*
- Associate pedigrees with some traits

Detection of hybrids

- Hybrids
  - Individuals whose recent ancestry includes both species
- Applies only to the nuclear genome
- Good baseline collections of both species
  - 1492 entries
  - 100 *JA*
  - ~1000 *JC*
- Requires formal analytical methods
  - First filial (F_1) detection
  - Advanced generation (BC_x and F_2 detection)
- Requires formal analytical methods
- NewHybrids Version 1.1 Beta 3*
  - Ran with a uniform prior 50,000 burn in 100,000 sweeps
  - Assigned to one of six classes
  - JC, JA, F_1, F_2, BC_1 to JC, BC_1 to JA

What we have already done:

- Developed a bombproof species-specific detection kit for the chloroplast a robust set of nuclear markers for butternut and Japanese walnut
  - Species assignment
  - Hybrid detection and classification
  - Cultivar definition and identification
- Developed 11 markers for cultivar identification in *C. mollissima*
- Genotyped 296 “Chinese” chestnuts and detected 10 major genetic groups

Mark Double, West Virginia University

Backcross orchard for assessment of host resistance combined with hypovirulence

W.L. MacDonald and M.L. Double (in conjunction with Fred Hebard and Sara Fitzsimmons, The American Chestnut Foundation®)

Six replicate plots containing 150 trees each have been established at the Plant and Soil Sciences Farm in Morgantown, WV to assess the interaction of host resistance and virulent/hypovirulent strains of *Cryphonectria parasitica*. Seeds have been planted annually from 2006-2011. As of September 29, 2011, overall survival was 82%, specifically:

Species	Percent Survival
American	87%
Backcross	88%
Chinese	95%
European	45%

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

Fred Hebard, The American Chestnut Foundation®

Current aspects of the backcross breeding program at Meadowview. At a meeting this summer to discuss the next steps following the Burnham breeding plan, the question was asked, 'how would TACF® augment their breeding stock with transgenic resistance, or any other new source of resistance to blight or Phytophthora root rot?' The goal of the breeding program is to enable the American chestnut to resume evolving on its own, as a wild species. The strategy is:

- First, American and Chinese chestnut trees are crossed with each other.
- The progeny from this first cross are then backcrossed to American chestnut.
- Each cycle of backcrossing reduces the fraction of Chinese genes by a factor of one half.
- Finally, backcrosses are intercrossed to yield progeny that will breed true. Burnham hypothesized that, with his experience in corn genetics, that this program should restore blight resistance to American chestnut. The current breeding program was jump started two generations using 'Graves' and 'Clapper', first backcross trees, from the 1920-1940 USDA breeding program. These trees were crossed with American chestnuts at Meadowview between 1989-1995 and the third backcrosses were made between 1994-2002. Hebard is about finished making third backcross F_2s, hoping that they will be done by 2012. The purpose of the breeding program is to dilute out the traits of the Chinese chestnut tree except for blight resistance. At the third backcross stage, if both parents have genes for blight resistance then there is a chance for recovering progeny that are homozygous for blight resistance. A different American parent is used in each step of the backcross to avoid inbreeding. This breeding strategy is very cumbersome and time consuming, but it is being replicated throughout the range of American chestnut through the state TACF chapters.
- Blight resistance is retained by inoculating progeny and selecting resistant.

In 2011, there are about 35,000 third backcross F_2s. This may be a peak number as they continue to rogue out trees after screening for blight resistance. How many B_3 lines are needed to avoid inbreeding? Hebard stated that using 20 B_3 lines (families) eliminates most inbreeding after intercrossing, enhancing the chance that a population will not collapse. The state TACF® chapters also add genetic diversity. Adding sets of 20 B_3-F_2 progeny from the state chapters on inbreeding effective population size, for one source of blight resistance, is effective. Restoration ecologists estimate that effective population size needs to be 50 to avoid immediate collapse from inbreeding depression and 500 for mutation to offset long-term erosion of genetic diversity by drift. Chapters also add adaptation to their local environment in addition to general genetic diversity.

Hebard indicated that the Meadowview breeding stock could be the base population for further improvement of American chestnut, although he feels that they have captured most of the genetic diversity with the current breeding program. If, however, there is not sufficient resistance, how is the breeding program further improved? The program can be augmented in several ways:

- Our populations will have to be persistent. This should be possible even if they have F_1 (intermediate) levels of blight resistance.
- They could be improved by planting stock with new sources of blight resistance nearby and using cultural manipulations to encourage seedling production.
- We might also wish to arrange for some pollination control and growth of seedlings in orchard settings.
- The basic breeding method becomes one of recurrent selection (with important modifications)

A more fundamental question is this—is the program working?

- Are we getting a lot of resistance at B_3-F_3?
- How are the trees doing out in the woods?

The F_1 between Chinese and American chestnut is intermediate in blight resistance in terms of canker size class. American chestnuts have large cankers, F_1s have intermediate size cankers and Chinese have small cankers. Some trees that have been recovered at F_2 have small cankers, better than the F_1. It was the intermediate levels of resistance that buffaloed the early breeders, causing them to cross the hybrids with Chinese to try and obtain higher resistance levels.

Rather than laboriously measure each canker, Hebard devised a 1-5 canker rating system (1=small cankers and 5=large cankers). Small cankers do not extend beyond the initial lesion; medium cankers extend beyond the initial lesion, usually only over part of its circumference, and do not become large and sunken, with abundant stromata; large cankers have a larger surface area than medium cankers, appear sunken in the fall after inoculation in the spring, and show abundant stromata of the blight fungus. This rating system is shown in the following table.

Qualitative System for Rating Blight Resistance in Chestnut.

Fungus Strain	**Canker Size after One Season & Resistance Rating**				
Ep155	Small-1	Medium-2	Large-3	Larger-3	Largest-3
SG2-3	Small-1	Small-1	Small-1	Medium-2	Large-3
Composite Rating = Sum of Two Strains minus One*	High-1	2	Intermediate-3	4	Low-5

* For more than two inoculations, subtract one minus the number of inoculations

The B_1F_2 trees behaved abnormally, with many cankers rated 1 or 2, rather than only a few, as has been seen repeatedly in the past, and their distribution was about the same as the B_3F_3 progeny, which was disappointing to Hebard. There probably will be more separation in canker size between F_1 and Chinese with time.

Number of Clapper B_3-F_3 seedlings in resistance classes after inoculation with two strains of the blight fungus in 2011, preliminary.

		Resistance Rating				
Cross	**N**	**1**	**2**	**3**	**4**	**5**
American	21		2	4	9	6
F_1	11	4	5	1		1

Chinese	32	25	4			
F_2	50	3	11	21	4	11
B_1F_2	173	33	49	59	18	14
B_1 x C	45	16	19	4	2	4
B_3F_3	614	97	150	220	99	48

The above data are preliminary; Hebard likes to wait a year before final assessments are made. Additionally, the data had not yet been analyzed by individual families. The purpose of the above test was to make selections between families.

The first field tests for restoration of American chestnut (*Castanea dentata*) in the Southern Region. Stacy L. Clark, USDA Forest Service, Southern Research Station; Scott E. Schlarbaum, Department of Forestry, Wildlife & Fisheries, The University of Tennessee; Fred V. Hebard, Staff Pathologist, The American Chestnut Foundation; Arnold Saxton, Animal Science Department, The University of Tennessee; John Blanton, David Casey, USDA Forest Service, National Forests in North Carolina, Asheville, NC; Barbara Crane, USDA Forest Service, Southern Region, Atlanta, GA; Russ MacFarlane, USDA Forest Service, National Forests in Virginia, Roanoke, VA; Jason Rodrigue, USDA Forest Service, National Forests in North Carolina, Asheville, NC; and, Jim Stelick, USDA Forest Service, National Forests in Tennessee, Cleveland, TN.

The objectives of this study (established in 2009) were:

- Determine differences in growth, survival, competitive ability, and blight resistance
    - Among putative blight-resistant families
    - Among breeding generations /parental species
    - Between two seedling size classes
- In representative forest conditions

Plots were established in: NC (USFS); TN (USFS); VA (USFS); IN (USFS); NC; PA; NJ; VA; WV; and WV (USFS).

Summary

- B_3F_3 seedlings are not behaving exactly like Americans, but are not similar to Chinese
- *Phytophthora* confirmed on all plantings
    - Worst symptoms at 2010 and 2011 plantings
    - 2009 shows little evidence for symptoms now
    - Likely coming from nursery seedling roots
    - They are developing a systematic sampling scheme for 2010 and 2011 plantings to test for effects on trees
- Blight and *Phytophthora* are likely interacting making blight resistance difficult to test

New findings about *Phytophthora* root rot:

- There appears to be a new species of *Phytophthora* associated with the root rot observed at the Augusta County Nursery, and in forest test plantings established dating back to 2009. According to Anagnostakis, the root rot might be incited by *Phymatotrichopsis ominvora*, the incitant of Texas root rot, rather than a species of *Phythophthora.*
- This species of *Phythophthora* may be more tolerant of cold conditions.

- To avoid having this new species of *Phythophthora* play havoc with the test plantings, they are considering immediately transition to containerized production of seedlings for outplanting.

Adam Lyon, University of Tennessee-Chattanooga

Investigating the shade tolerance of *Castanea dentata*, *C. mollissima*, and their hybrids. Lyon is an undergraduate student at UT-Chattanooga, working in the University Honors Program under the direction of Drs. Hill Craddock and Jennifer Boyd. Lyon investigated light availability of trees from the TACF® breeding program when planted in forest settings. One concern is that because the hybrids do have some Chinese chestnut ancestry, they may be more adapted to the high light environments associated with the Chinese chestnut, and not be as shade tolerant as the American chestnut. A second concern is that by conducting breeding efforts in greenhouse and orchard-like settings, artificial selection may be occurring for hybrids that are more adapted to high-light environments, and that may not be as shade tolerant as the American chestnut. In order make it to the next generation in the breeding program, individuals must survive to reproductive age. Lyon attempted to answer the research questions:

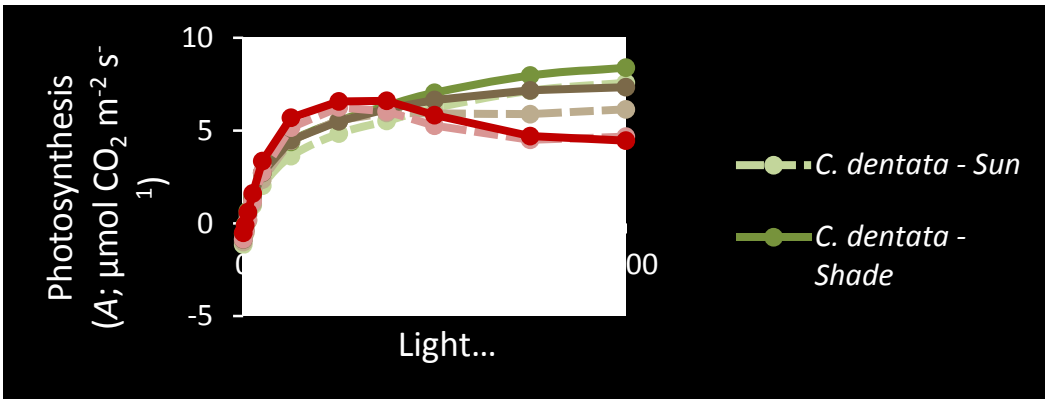
- How shade tolerant is the American chestnut? Because hybrid chestnuts are 94% American, the answer to this question could provide insight for guiding restoration site selection and management.
- How do hybrid chestnuts compare to their ancestors? One of the inherent assumptions of the breeding program is that, with the exception of blight resistance, hybrids will 'act' like the American chestnut, and this too could have important implications for restoration site selection and management.

To address these questions, Lyon quantified factors indicative of shade-tolerance of 1.5-year-old pure American, pure Chinese, and hybrid chestnuts seedlings growing in well-watered and fertilized pots outside the UTC greenhouse. In Spring 2010, he propagated 48 individuals total of each of these chestnut types. Half of the individuals of each chestnut type were grown in full ambient sunlight and the other half were grown under a 40% shade-cloth canopy. During the summer of 2011, Lyon assessed survival, growth and leaf-level physiology of all studied individuals.

Species classified as shade tolerant can germinate, grow and survive in low light, while shade intolerant species require high irradiance for their germination, growth and survival. The shade tolerance of a species can be related to its successional status. Colonizing or pioneer species are commonly shade intolerant, while later successional species are commonly shade tolerant. Although Lyon started with 24 American chestnuts in each light treatment, by the end of summer 2011, only 19 sun plants and 16 shade plants remained. Survival was somewhat greater with increased light availability. He also found that both seedling height and root crown diameter significantly increased with increasing irradiance. Observably, American chestnut also had greater stem diameter in sun than shade. These results indicate that American chestnut experiences enhanced survival and growth with increased irradiance. But many shade tolerant species, such as red maple, rarely exhibit significant growth with high light availability. Lyon's data showed the average response of leaf-

level photosynthesis to increasing irradiance. For American chestnut grown in full sun, compared with those grown in 40% shade, the levels of photosynthesis were similar. However, at very low light levels, such as those that might exist in a deciduous forest understory, data suggest that shade-grown American chestnut may exceed American chestnut grown in full sun in terms of leaf-level photosynthesis; this is characteristic of leaves adapted to shade. American chestnut had similar maximum rates of light-saturated photosynthesis whether grown in full sun or in shade. Also, the A_{max} values that he observed were within the range of those reported for shade-intolerant trees. In contrast, respiration rates were significantly greater in American chestnut grown in full sun compared with shade-grown American chestnut. Like respiration, both the light-compensation point (which is the light level at which photosynthetic carbon gain offsets respiratory losses) and the light-saturation estimate (which is the irradiance level at which light-saturated rates of photosynthesis are attained) were significantly lower when American chestnut was grown in shade than when it was grown in full sun. Data were all within the low ranges reported for shade tolerant species, and they indicate that acclimation of photosynthetic processes does occur with decreasing irradiance in American chestnut. This also has been considered to be an indicator of shade tolerance. The differences in quantum efficiency that we observed between light treatments were not significant, suggesting that the seedlings converted light to CO_2 as efficiently in full sun as in shade. The lack of significant increase in this variable is suggestive of being less shade tolerant. What do these results suggest regarding the shade tolerance of the American chestnut? Lyon's results are in agreement with earlier research primarily from the University of Ohio that American chestnut be classified as intermediate in shade tolerance. It clearly does well in shade, but could take advantage of canopy openings as well. His data suggests this classification is based on a number of factors. The American chestnut is clearly able to survive in relatively shady environments, as evidenced by the many sprouts in eastern deciduous forest understories and its historical ability to grow up through the shady understory into the forest canopy. However, Lyon's results indicate that American chestnut experiences enhanced survival and growth with increased irradiance. Many known shade tolerant species, such as red maple, rarely exhibit significant growth with high light availability. In addition, the leaf-level photosynthetic variables that he analyzed provided a mixed message in terms of the shade tolerance of American chestnuts

Lyon's second objective was to see how hybrid chestnut compares to its ancestors in terms of shade tolerance? Overall, survival when grown in full sun or shade was greater among hybrid and Chinese chestnut than Americans. Across chestnut types, height, stem diameter and root diameter were all significantly greater in sun vs. shade. However, only height significantly differed between chestnut types. Specifically, American and hybrid chestnut were significantly shorter than Chinese chestnut. This suggests that hybrid growth most resembled that of the American chestnut.



In terms of physiology, from data in the graph above, the curves for American chestnut grown in sun and shade look similar. The curves for Chinese chestnut grown in sun and shade also appear similar to one another. Most notably, Chinese chestnut exhibits photoinhibition at higher irradiance levels which is a light-dependent down-regulation of photosynthesis. Although it may seem ironic, photoinhibition is thought to be a strategy in plants adapted to high sunlight that enables them to avoid or dissipate excess light energy, because light that exceeds the ability of a leaf to utilize it can cause photodamage. Lyon suggests that photoinhibition could have helped to enhance the growth of Chinese chestnut relative to American chestnut in the relatively high-light experiment. In contrast to the American and Chinese chestnut, hybrid chestnut appeared to respond differently to increased light when grown in shade compared to when grown in full sun. Specifically, shade-grown hybrid chestnut exhibited responses similar to the American chestnut, but hybrid chestnut grown in full sun exhibited some of the photoinhibition of the Chinese chestnut. This suggests that hybrid chestnuts may have an increased ability to acclimate in this regard. By analyzing the data, Lyon was able to compare variables responsible for these different observed light responses among chestnut types, and there were significant differences in both maximum photosynthesis and respiration. Both were significantly greater in both the American and hybrid chestnut than in the Chinese chestnut, suggesting that overall gas-exchange rates of hybrids resemble the American chestnut. There were no significant differences in the light compensation point or light saturation rate among chestnut types either within or across light treatments. In contrast, quantum efficiency was significantly influenced by the interaction of chestnut type and light treatment. Specifically, the quantum efficiency of Chinese chestnut was not influenced by light and was always less than the quantum efficiency of the American chestnut. But, the quantum efficiency of both American chestnut and hybrid chestnut was significantly greater in shade than in sun although hybrid chestnut quantum efficiency was the same as the low values exhibited by Chinese chestnut when hybrids were grown in full sunlight. When grown in shade, hybrid quantum efficiency was as high as that of the American chestnut. These results suggest that for hybrid chestnuts there is a great amount of acclimation in this factor to light availability.

Lyon offered conclusions about the possibility of introducing hybrid chestnut to the forest understory. He suggests that hybrids exhibit a degree of shade-tolerance that is more intermediate than the American chestnut. This may be for several reasons. Overall, growth of hybrid chestnut across light treatments was similar to the American chestnut, suggesting that it can be tolerate shade but also respond positively to increased irradiance. But, hybrid chestnut

exhibited a greater ability to acclimate in regard to photosynthetic light-response factors when grown in full sun compared with shade compared to the American chestnut. In particular, hybrid quantum efficiency was more like that of the American chestnut when grown in shade but was more like that of the Chinese chestnut when grown in sun. This suggests that hybrids might have inherited genes that influence light response from both their American and Chinese ancestors. Lyon also suggests that hybrids may have an advantage over Americans if light became more available in the understory because hybrids exhibited some photoinhibition when grown in full sun like the Chinese chestnut. Lyon noted throughout the past summer that leaves of the American chestnuts grown in full sunlight appeared mottled and evidenced some white discoloration, suggesting that perhaps leaves were experiencing damage due to long-term high-light exposure. Lyon suggests that future research could investigate this occurrence more intensively. Furthermore, he suggests that related experiments utilizing natural light gradients in a field setting be conducted to examine comparative hybrid chestnut grown in the context of 'real world' limitations like water and nutrient availability.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

Crosses made in 2011 concentrated on introducing gall wasp resistance into commercial chestnut cultivars and American chestnuts, and blight and ink disease resistance into Ozark chinquapins. For gall wasp and blight resistance, *Castanea henryi* (Chinese chinquapin) pollen was collected and saved (dry and cold) for use on Ozark chinquapins from Oklahoma and Arkansas, on an American chestnut from Connecticut, and on ramets of 'Colossal', 'Bouche de Betizac' and 'Eaton'. There was good nut set with the chinquapin and 'Eaton' crosses but none with 'Colossal' or 'Bouch de Betizac' and there was very poor nut set on the American used. Richard Jaynes said in his 1961 dissertation that he found good evidence of pollen incompatibility between American and *henryi* and between *seguinii* and *henryi*. This appears also to be the case with the two commercial chestnuts. Crosses of Ozark chinquapins with a Japanese timber tree with good blight and ink disease resistance yielded 82 nuts. Seedlings from these will be grown and back-crossed to chinquapins when they flower.

John Carlson, Penn State University

The chestnut genome project. This project was supported by the Forest Health Initiative (http://foresthealthinitiative.org) to evaluate the role of biotechnology in addressing forest health challenges, starting with American chestnut restoration. The Chestnut genome sequencing project builds upon the results of the previous NSF-sponsored "Genomic Tool Development for the Fagaceae" project (www.Fagaceae.org) and upon breeding materials developed by The American Chestnut Foundation®. Direct participants in the project included: John Carlson; Stephan Schuster; Webb Miller; Charles Addo-Quaye; Lynn Tomsho; Daniela Drautz; Lindsay Kasson; Tyler Wagner; and, Nicole Zembower at Penn State University, with Meg Staton, Abdelali Barakat and Bert Abbott at Clemson University.

Genomics

- Whole genome sequence of blight-resistant Chinese chestnut
- Genome resequencing of American chestnut
- Discovery of host candidate resistance genes

Genetic technologies and breeding

- Refine genetic maps and QTL for blight resistance
- Map QTL for *Phytophthora cinammomi* resistance
- Develop DNA markers to aid selection in breeding programs
- Develop early blight screening protocol

Transgenics and propagation

- Refine somatic embryogenesis and transformation protocols
- Test new candidate genes in transgenic plants
- Screen transgenic plants in lab and field tests
- Develop strategy for deploying transgenic trees in breeding and ecological restoration programs

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 *C. parasitica* and other important traits, genome sequences also will be produced for American chestnut, for other genotypes of Chinese chestnut, and for other *Castanea* species. It is hoped that the broader impact of this project will be to demonstrate the power of genomics in addressing the forest health and ecosystem restoration.

*Progress, to date***.** Genome sequencing has progressed to over 22X depth (17.8Gb) of simple-end and paired-end reads from the 454 platform, plus 43,143bp of BAC end Sanger sequences (covering the minimal tiling path of BAC clones from the chestnut physical map) plus 37.5 Gb of short reads from Illumina sequencing platform. The most recent assembly of the genome sequence (build number 12) place 925Mbp into 1,147,939 contigs, of which approximately 587 Mbp could be further assembled into 51,766 scaffolds averaging 52Kbp in length. Thus, more than the estimated genome size of 800Mb for Chinese chestnut (Kremer et al 2007) appears to be assembling, suggesting high levels of genomic heterozygosity or contamination. Although the current assembly is far from complete, over 86% of the 48K Chinese chestnut transcript contigs from the *Fagaceae* genomic tools project aligned with 98% or greater identity to the genome contigs, suggesting very good coverage of expressed genes. Annotation of the current genome build using gene search algorithms and BLAST to Phytozome identified over 550 disease resistance genes to date, including 13 pathogenisis-response proteins, 40 disease resistance family proteins (TIR-NBS-LRR), and 80 leucine-rich repeat family proteins.

The largest gene identified in the preliminary Chinese chestnut genome assembly was:

Homolog of AT1G67120 (NP_176883.4), AAA ATPase, von Willebrand factor type A domain-containing protein, with nucleoside-triphosphatase activity.

- Transcript length: 43,203 bases
- Number of Exons: 71
- Scaffold ID: *scaffold01252*

To date, over 550 resistance genes have been identified.

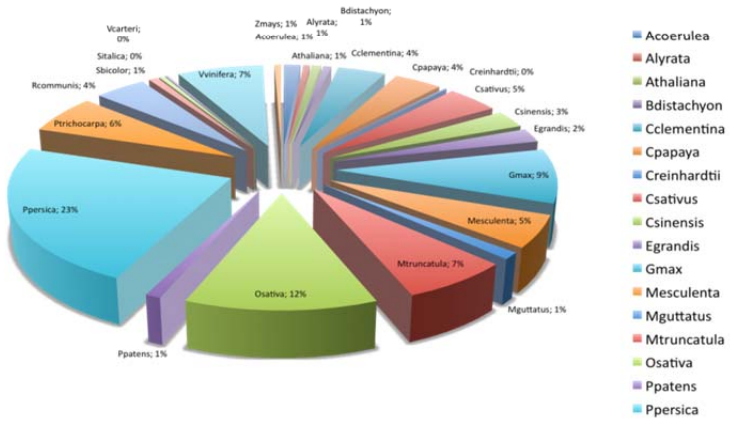
Classes of disease resistance genes found	Number
Pathogenesis-response proteins	13
Disease resistance family protein (TIR-NBC-LRR)	40
Leucine-rich repeat family proteins, total	80

Genes differentially expressed in *C. mollissima* vs C*. dentata* cankers	433

To resolve the genome assembly issues, additional sequencing of a new genomic DNA preparation from a single *Castanea mollissima* cv 'Vanuxem' ramet is underway, using the new extended read length 454-technology. Resequencing of additional chestnut genomes is also underway. Their first resequencing of additional chestnut genomes also is underway. Their first resequencing run, to 40X depth on the Illumina platform, was conducted with the American chestnut genome used to construct the BAC library for American chestnut. Genomic DNAs for resequencing of *C. henryi* and *C. sequinii* were provided by the recent visit to China by Bill Powell's group, as well as from the CAES collection by Sandra Anagnostakis. The genome resequencing data will be assembled by mapping of sequence reads onto the Chinese chestnut reference genome, once completed.

Carlson's group just sequenced four pools of BAC clones at CUGI from the physical map which represent contigs at the three blight resistance QTL. The physical map contigs for the QTL in LG-G (BAC pool A) and LG-F (pool B) were 5.13 Mb and 2.50 Mb in length, while the two BAC pools for the LG-B QTL covered 4.81 Mb (pool C) and 5.33 Mb (pool D). They obtained approximately 23X depth of sequence using the new extended read lengthy 454, with reads averaging 493 bp. Assembly by Meg Staton produced 572 contigs with an N50 of 14,809 bp for pool A, 857 contigs with an N50 of 14,447 bp for pool B, 982 contigs with an N50 of 10,895 for pool C and 828 contigs with an N50 of 10,516 for pool D. Over 86% of the Sanger BAC ends, the sequenced BACs in the contigs appeared in the assemblies. Another indication of good coverage in the assemblies was that 95% of the 454 reads were placed in contigs. Annotation revealed a total of 128 matches of *Arabidopsis* genes to the BAC contig sequences from all three QTL. BLAST search of the BAC contig sequences with the Chinese chestnut transcriptome produced a total of 246 matches, from a total of 48K transcript contigs. Many of the *Arabidopsis* genes aligning with BAC contig sequences were similar to all four QTL contigs, including amino acid permease, glycosyltransferases, beta-galactosidases, cysteine-rich flk receptor-like proteins, zinc finger proteins, transcription coactivators, NAD kinases, phosphatases, pentactricopeptide, repeat (ppr) proteins, protein kinases, wd40 repeat proteins, wall-associated kinases, alpha/beta-hydrolases, auxin response factors, glutamate receptors, callose synthase, transmembrane recptors, cysteine pepsidases, glycosyl hydrolases, leucine-rich repeat protein kinases and proline-rich extension-like receptors. However, few classical *Arabidopsis* PR or pathogen defense genes have been identified in the sequence at this point.

Best matches of proteins from the chestnut genome assembly are to peach and other related species



Best matches:

- peach, 23%
- rice, 12%
- grapevine, 7%
- Eurosids 1 species, 56%

BLASTx alignments to model plant genomes in Phytozome

Dana Nelson, USFS-Southern Institute of Forest Genetics

Disease resistant mapping and candidate gene selection. Chestnut markers and mapping:

- Initial funding was secured a few years ago by NSF (Ron Sederoff was the PI)
- Current funding by Forest Health Initiative (FHI). There are three groups working on this project:
  - Biological sciences group
    - coPI: Dana Nelson, John Carlson, Bill Powell, Scott Merkle
  - Social/Environmental—the outcomes may produce genetically modified plants for outplanting (Heisenbuttel)
  - Regulatory/Policy to cover the genetically modified tree testing, etc. (McCord, Costanza)
- Personnel on chestnut markers and mapping
  - Tom Kubisiak (SIFG) retired in February this year. Nelson is trying to pick up some of Kubisiak's work.
  - Bode Olukolu, Post doc at Clemson has moved to NC State in June, working on corn.
  - Tatyana Zhebentyayeva (Clemson) started this month.
  - Laura Georgi (TACF) started this month.
  - Fred Hebard and Sara Fitzsimmons (TACF®) phenotyping, leaves, etc.
  - Meg Staton (Clemson) is EST sequencing/bioinformatics support.
  - Dennis Deemer, Chuck Burdine (SIFG) DNA preps, SSRs (Deemer left this year too).

NSF Resources, many resources were developed around *Castanea*, mostly ESTs

- ESTs
  - Many libraries were made, much sequencing (mostly done by Carlson, et. al)
  - Genetic maps were made with SNP and SSRs
    - SNPs & SSRs from ESTs
    - 2 crosses (Nanking x Vanuxin & Mahogany, n=179 & 158)
    - American mapping population (GMBig x Horn, n=166) was done by Hebard.
    - SNPs on F_2 mapping population (Mahogany)
- BACs
  - Several libraries made, two were used for physical map.
    - High information content fingerprinting

Mapping results

- Genetic
  - Consensus Chinese map
    - 1156 markers (840 SNPs + 316 SSRs); 743 cM
  - American map was found to be highly co-linear to the Chinese map.
    - 281 markers; 738 cM
  - F_2 map; 12 linkage groups were clarified and 3 QTLs were verified.
    - 463 markers; 686 cM
- Physical (most of this information is found on the www.fagaceae.com site)

- 1377 contigs; 12,919 singletons; 18X coverage
- 1026 markers anchored to genetic map

The three QTLs that are highly significant are on linkage group B, F and G. Data from the two parents (F_1 trees) and how they react to two fungal isolates is listed below.

Characteristics of blight QTLs

	R4T31		R4T52	
Linkage Group	**SG 2-3**	**Ep 155**	**SG 2-3**	**Ep 155**
B	a^C	a^C	a^C	a^C
F	a^C		a^C	
G		a^C		a^C
E			d^C	d^C

a= additive effect of resistance
d= dominance effect of resistance
C= Chinese allele provides resistance

On linkage group E, there is some evidence that there is another QTL and it appears to segregate from only one parent, effective against both isolates. It appears to be dominant and not additive.

Nelson showed a Chinese chestnut genetic map. There are 1126 markers covering 743 cM with 3 blight resistance QTLs from F_2 map and 2 ink disease resistance QTLs from B_1 map.

Moving forward, they wanted to increase the density of markers and the resolution with respect to the QTL position using larger populations. The initial SNP mapping had 1536 SNP array based on Chinese ESTs. This has been increased to 5129 SNPs; this should be completed in 2011. This will be used to map larger populations, an expanded F_2 population. There will be 210 individuals that have been phenotyped by Fred Hebard. Sara Fitzsimmons has a large B_3 population—about 750 individuals in 11 B_3 families that have been inoculated with both fungal isolates. There is now DNA and phenotypic data on all the individuals. Other populations will be genotyped as well (the DNA is ready).

- Clapper B_2 (n=79)
- Nanking B_1 (n=103)
- Mahogany B_1 (n=62)
- Nanking B_1 (GL158) (n=50), *Phytophthora* mapping

Working with Fred Hebard over the last two years, they have been sampling B_3F_2 populations from Meadowview.

- DNA ready; 33 families, (n=6500 trees)
- DNA in process; n~4000
- Need more cost efficient means to genotype this large a population
  - Candidate SNPs, SSRs
  - Genotyping by sequencing, a new technique—prices as low as $10/ sample

<u>5K SNP array design</u>

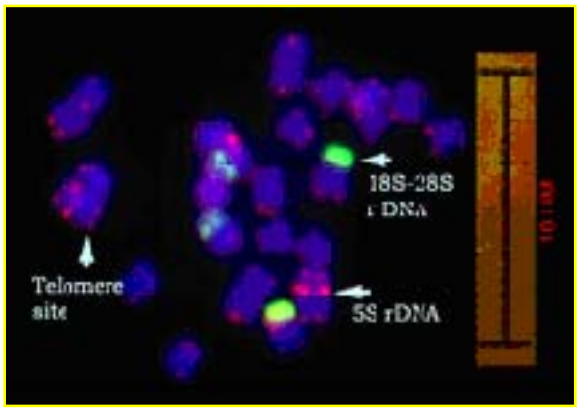
CCall_v2_contigs→ 5069 SNPs passed (964 were validated) covering 2727 contigs
AC454_v3_contigs→ 3199 SNPs passed (264 were validated) covering 1945 contigs
Totals→ 8268 (1228) and 4672

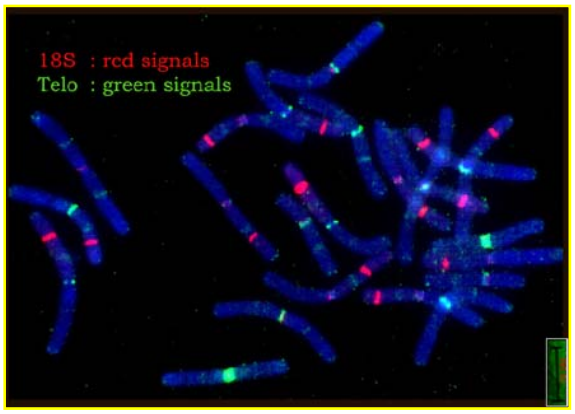
	SNPs	Bead Types
Chinese	3102	3600
American	2027	2400
Total	**5129**	**6000**

Candidate Gene list. Working with the FHI group, they looked at a number of sources of information, including QTL map position to identify candidate genes for resistance—genes that have different sources evidence that would be involved in gene resistance and collocated in the QTL. There are 42 on the current list. Nelson is working with Bill Powell (SUNY-ESF) and Scott Merkle at (University of Georgia) to evaluate these genes. A partial list is seen below.

CCcontig	Uniprot_BestHit	Linkage_Group	cDNA_status	BinaryVector	BV_status	TransPipe
CCall-contig8901_v2	**beta-1 3 glucanase**	?	Cloned&sent	pFHI-B13Gluc	received	SUNY-ESF
CCall-contig2586_v2	**CBS domain protein**	?	Cloned&sent	pFHI-CBS1	received	SUNY-ESF
CCall-contig11269_v2	**UDP glucosyltransferase**	B, G	Cloned&sent	pFHI-UDP	received	UGA
CCall-contig8443_v2	**Thaumatin-like protein**	G	Cloned&sent	pFHI-Thau	received	UGA
CCall-contig9278_v2	**DAHP synthase (DHS1)**	G	Cloned&sent	pFHI-DAHP	received	SUNY-ESF
CCall-contig8996_v2	**Acid phosphatase**	G	Cloned&sent	pFHI-AcPhos	received	UGA & SUNY-ESF
CC454-contig42836_v2	**Laccase/diphenol oxidase**	B	Cloned&sent	pFHI-Lac1	received	SUNY-ESF
CCall-contig18406_v2	**Proline-rich protein**	G	Cloned&sent	pFHI-PRP1	received	SUNY-ESF
CCall-contig19527_v2	**Ethylene-response transcription factor**	F	Cloned&sent	working	waiting	for UGA
CC454_contig2466_v2	**Unknown function**	E	working	working	waiting	TBD
CCall_contig39658_v2	**Lipid transfer protein (LTP)/proteinase inhibitor**	G	working	working	waiting	TBD
CCall_contig_2055_v2	**Lipid transfer protein SSH**	?	Cloned&sent	working	waiting	TBD
CCall_contig4992_v2	**Cysteine proteinase inhibitor**	E	Cloned&sent	working	waiting	TBD
CC454_contig41915_v2	**Allene oxide cyclase (AOC)**	?	working	working	waiting	TBD

Cytogenetics. Nelson reported on cytogenetics work by Nurul Faridi, SIFG at Texas A&M University. Faridi has done a lot of work on pine and Nelson has convinced him to look at chestnut. The chestnut genome is ~800 MB while the pine genome is 20 Gb (pictured below). Faridi found multiple 18S sites and one 5S rDNA sites in root tips from American chestnut. When looking at an open-pollinated family from the cultivar AU-Croppper, Faridi has determined that there is heterozygosity at the major 18S site locus. He also has found this in other Chinese chestnut lines.



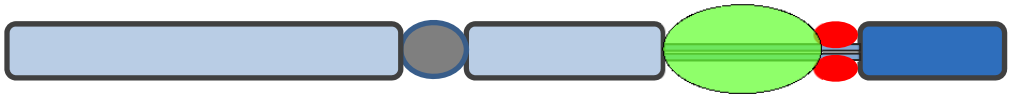


Chestnut 1C ~800 Mb

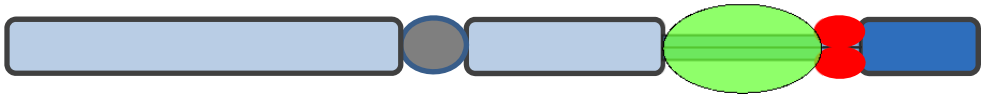
Pine 1C > 20 Gb

NOR chromosome variants in Chinese

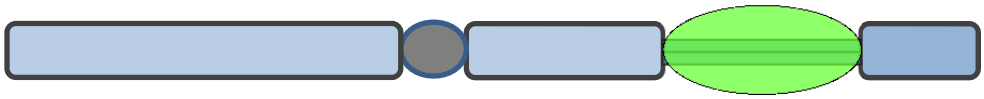
A. with 5S; large heterochromatic



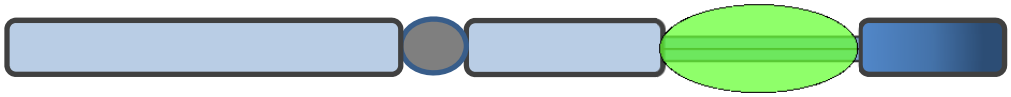
B. with 5S; medium heterochromatic



C. no 5S; medium, non-heterochromatic



D. no 5S; large heterochromatic



NOR chromosome in American

Faridi has carefully characterized the American chestnut chromosome nuclear organizing region (NOR) and he has a definitive karyotype that is uniform in American chestnut. This varies from the four different types of NOR in Chinese chestnut.

Fairidi, in previous work, found translocation between American and Chinese chestnut. They want to take the BAC clones and integrate them with the genetic maps and use FISH to map out the chromosomes.

Early screening at the Resistance Screening Center in Asheville, NC. Nelson is interested looking at early screening of backcross chestnuts. The center primarily screens for fusiform rust on southern pines. Secondarily, they screen for pitch canker on pines. Chestnut now has been included. The screening of chestnuts included 2 and 3 year-old seedlings from Bob Leffel from the PA-TACF® chapter—all B_1 families. There were several sources of resistance, with one-to-a-few B_1 families per resistance source. There was a target of 10 seedlings per family, replicated three times. Two *C. parasitica* strains were used; the upper site was inoculated with Ep 155 and the lower site was inoculated with SG 2-3. Canker length, girdling rate and stem diameter were measured. The results were:

- Very similar tree size between 2 and 3 year-old trees
  - Better growing protocol was used for younger trees
- Very similar disease development between ages
- Correlation between isolates on same tree was same on both age trees (r=0.30)
- Correlations between isolates' canker length or area on same tree by tree age

Conclusions of resistance screening:

- Promising for large scale small stem screening
- Need larger family sizes to accurately rank resistance
  - Used:
    - Age 2: 6 to 19 seedlings per family
    - Age 3: 10 to 30 seedlings per family
  - Recommend at least 30 seedlings (3 reps of 10)

Gary W. Micsky, Penn State Cooperative Extension in Mercer County (submitted report)

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

Methods:

- Training workshops and field experience
- Extension newsletters, press releases, woodland owner association newsletters
- Grower/Site evaluations
- Pest Surveys
- Evaluation Process:
- Number of 2011 research/test orchards established (N=2)
- Number of 2011 on-site test plantings established (N=54)

- Volunteers who learn to identify reproductive structures and correctly prepare female flowers for pollen exclusion and eventual controlled pollination (N=4)
- Success of pollination efforts (yield = 67 F_1cross nuts)
- Number of trained volunteers (44) and volunteer hours (155) as of 9.23.11
- Chestnut vigor/survival on site assessment plots

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 1-7, 2010 Chestnut Seed Harvest Collected and processed 123 BC_4 cross and approximately 5000 open pollinated American chestnut seed for use in local and TACF plantings in 2011. Volunteers (N=12) Volunteer Hours: >24
- November 3, 2010 NE 1033 Multi-state Research Poster 2010 PSU Extension Annual Conference & JCEP Association Meetings
- November 6, 2010 PA-TACF Fall Meeting, Mercer County Extension Office, hosted and secured funds to offset travel expense for Dr. Lynne Rieske-Kinney ($200 sponsorship from Mercer County Woodland Owners Association)
- March 5, 2011 "American Chestnut Site Selection and Aftercare Workshop" Tionesta, PA 20 participants
- March 14, 2011 Asian Gall Wasp gall collection in support of Dr. Lynn Riske-Kinney's research: Monitoring the occurrence of introduced and native predators/parasites of the Asian Gall Wasp. Volunteers: (N=6) Volunteer Hours: 18
- March 15, 2011 Plant 100 Haun OP seed in pots for PA-TACF use Volunteers: (N=2) Volunteer Hours: 6
- March 26, 2011 "American Chestnut Site Selection and Aftercare Workshop" Mercer, PA 38 participants
- April 27, 2011 US Army Corps of Engineers plant 5 test plots at Shenango Lake properties Ranger Staff (N=2) Hours: 8
- April 29, 2011 Freeman Tree Farm, Knox, PA Plant 100 BC_4 seed returned from Joe Jeffers/Paul Sisco *Phytopthora cinamomi* experiment Volunteers: (N=4) Volunteer Hours: 54
- May 19, 2011 "Ohio River Watershed Challenge-"Climate Change & Native Species" American chestnut station. Students from western PA high schools explored the past, present, & future of American chestnut. 65 participants - 5 volunteer hours
- June 11, 2011 Freeman Tree Farm June 25, 2011 Supplemental Chestnut Gall Sampling (N=2) Volunteer Hours: 3
- June 30, 2011 Place Pollen Exclusion Bags Volunteers (N=2) Volunteer Hours: 3
- July 6, 2011 Pollen Collection/Processing Volunteers (N=2) Volunteer Hours: 3
- July 8, 2011 F1 Pollinations Volunteers (N=2) Volunteer Hours: 2
- July 19, 2011 Supplemental gall collection for Dr, Lynne Rieske- Kinney Volunteers (N=6) Volunteer Hours: 6
- July 12, 2010 Pollination Demonstration for Greenville Record Argus

- September 19, 2011 Harvest F_1 Seed Volunteers (N=1) Volunteer Hours: 1
- September 22, 2011 Harvest F_1 Seed Volunteers (N=2) Volunteer Hours: 3

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

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

Update on JGI Genome Sequencing Project. The JGI released version V2 or the *C. parasitica* genome assembly to the public in March 2010 (http://genome.jgi-psf.org/Crypa2/Crypa2.home.html)

Re-sequencing of the genome of *C. parasitica* strain Ep 146. The Nuss laboratory arranged to have the Ep 146 genome sequenced to ~ 11 X coverage (486.6 Mbp) using 454 sequencing technology at the New Mexico State University Sequencing Center in collaboration with the Dawe laboratory. Raw reads (average length of 384 bp) were individually mapped onto the 26 scaffolds comprising the version 2 Ep 155 reference genome sequence within a searchable genome browser and independently assembled into 3600 contigs.

In total, more than 100,000 polymorphisms between these two strains were found. However, closer analysis showed that only 3,681 of these were located in predicted coding regions. Furthermore, only 2,079 of these were predicted to result in non-synonymous changes to a protein product. In light of the analysis produced during the study of Ep 155 that predicted 11,184 gene models (JGI v2.0), there would only appear to be an approximately 20% chance of any given gene containing a single non-synonymous change between Ep 155 and Ep 146. The Ep 155 and Ep 146 genomes were compared to look for clustered polymorphisms that represented candidate vegetative incompatibility genes. Alleles for *vic2*, *vic4*, *vic6* and *vic7* were identified and disrupted as described in the paper by Choi, et.al (Choi, G. H., Dawe, A. L., Churbanov, A., Smith, M. L., Milgroom, M. G., and Nuss, D. L. Molecular characterization of vegetative incompatibility genes that restrict hypovirus transmission in the chestnut blight fungus *Cryphonectria parasitica*. Genetics, *In Press*).

The genome of *C. parasitica* tester strain EU55 is currently being sequenced at NMSU in order to identify the *vic1* and *vic3* loci.

Overview. When Nuss first came to a NE-1033 meeting, there were two things that grabbed his attention. The first was double-stranded RNAs. Were they viruses and did they actually cause hypovirulence? The path to answering those questions was to isolate the viral RNA, cDNA clone it and in the case of mycoviruses, they have an additional problem in that they have an exclusive intracellular lifestyle, so you cannot grind up, isolate the virus and initiate an infection. The way to finish Koch's postulates in this case was to make an infectious cDNA clone. Gil Choi was instrumental in taking the cDNA clone and sequence information and Choi made the infectious cDNA clone that then allowed them to show conclusively that these viruses are responsible for hypovirulence. Having an infective cDNA system has allowed them to have an experimental system that has served them very well over the years. Later, they were able to

transfect the RNA in. Over the years they have been able to identify some aspects of the virus function of proteins.

In this regard, Ken Jensen will discuss p48. Roni Shapira showed from *in vitro* work that p48 is a protease that cleaves itself and Fuyou Deng showed that p48 is required for initiation of viral RNA replication but not for ongoing viral RNA replication. Those studies have been extended and Jensen will report on some interesting but still confusing results.

The second thing that interested Nuss was vegetative incompatibility (*vic*) that leads to programmed cell death. Virus transmission is impeded. This has implications for transmission of viruses in the field. In a diverse vegetative incompatibility profile, there will be difficulty getting the virus into the native population of the fungus. A number of things conspired over the last few years so that they were able to identify *vic* genes. Michael Milgroom and coworkers developed 64 *vic* tester strains that allowed strains of interest to be genotyped. Tom Kubisiak and others put together a genetic linkage map. JGI was convinced to do the genome sequencing. The idea was that if you sequenced another strain that differed at these vegetative compatibility loci you might be able to identify *vic* gene candidates by looking for regions of sequence polymorphism guided by the genetic linkage map. The other thing that happened was that Angus Dawe's Institution obtained a 454 sequencing machine and they re-sequenced Ep 146. Gil Choi came back to the lab and Nuss was able to start the project because he had a two-year window in between administrative duties.

Ep 146 was shown to differ from Ep 155 at *vic* 2, 4, 6, 7 as shown below:

vic genotype for *C. parasitica* strains EP155 and EP146

vic loci	**1234-67**
Ep 155	2211-22
Ep 146	2112-11

If Ep 146 is sequenced, the plan was to take those reads and layer them onto the reference sequence (Ep 155), and then use the approximate position based on the linkage map, and correspond to the sequence scaffold, and look between the markers where there might be a level of polymorphism. That was what they saw for *vic* 2. The corresponding allele can then be sequenced.

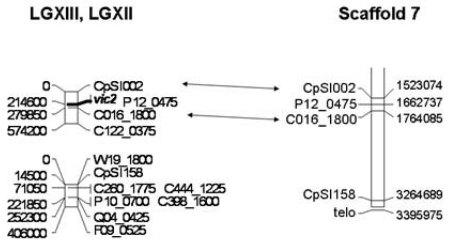
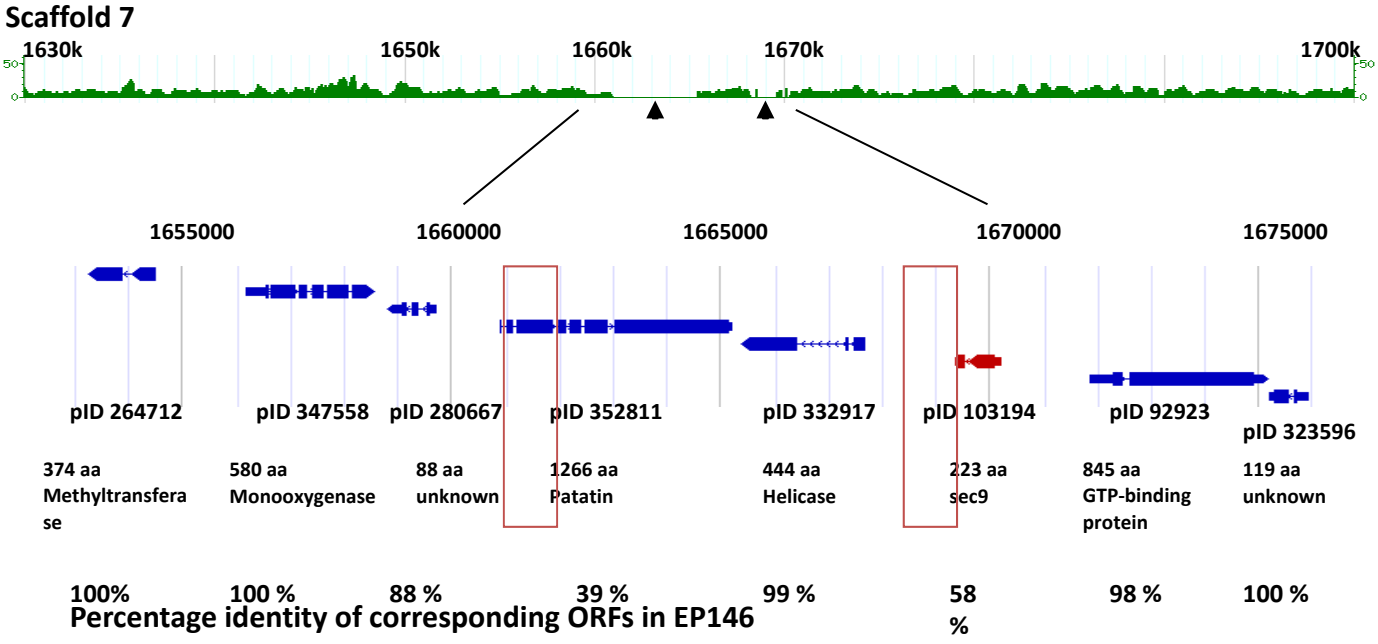


Fig. 2A

Two polymorphic ORFs at *vic* 2 were found—one looks like a protein storage protein and another small ORF that codes a secretory protein as seen the diagram below. Outside of the regions of polymorphism, there is 88% identify—very few SNPs, very little heterogeneity.

Islands in between the areas of polymorphism have been conserved. Nuss commented that it is amazing how these polymorphisms arose and they seemed to be fixed in the population.



*vic*4, shown below, was found to be very interesting. When they found where it is located, it turns out that it is not polymorphic but idiomorphic alleles. They are entirely different genes. In Ep 155, there is a kinase and it is completely and surgically replaced by another gene that has characteristics of heterokayon incompatibility genes.

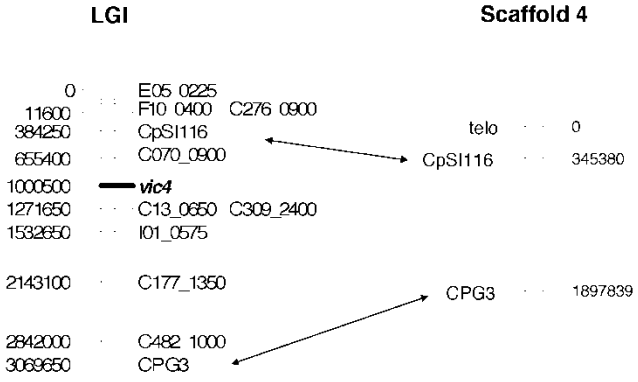
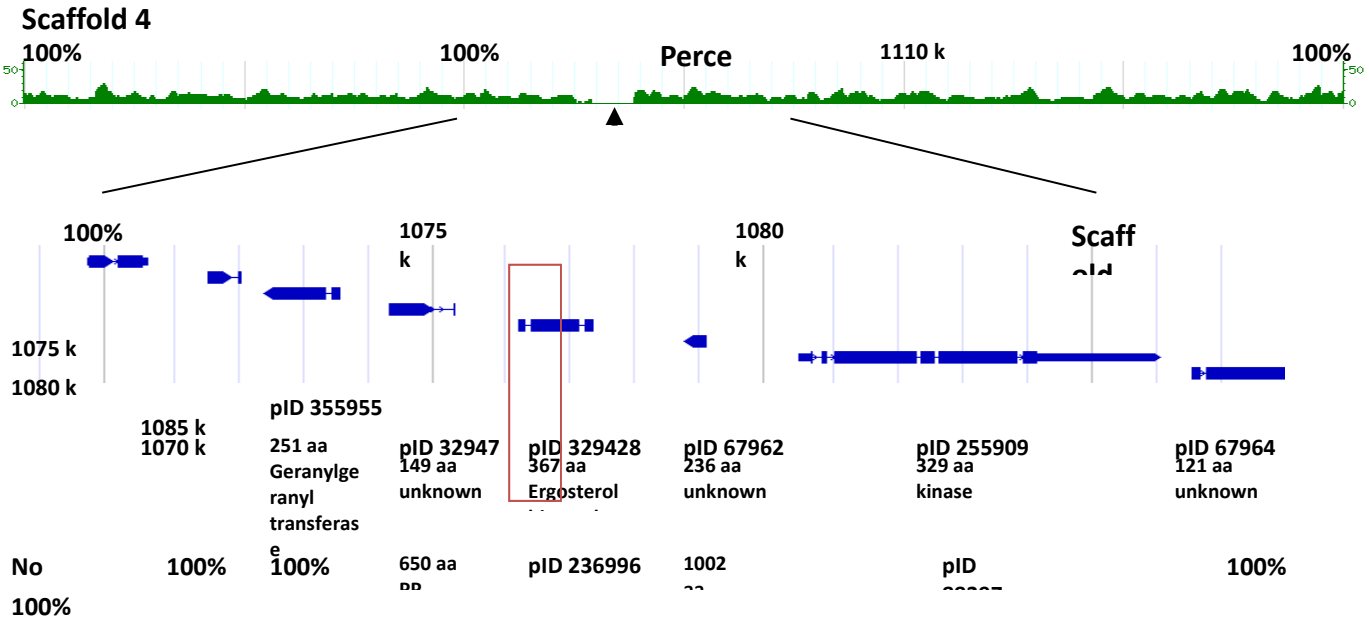


Fig. S3



*vic*7, on scaffold 6, has hallmarks of a heterkaryon compatibility gene. Its level of polymorphism is quite small so it is pretty well conserved.

*vic*6, on scaffold 3, contains a polymorphic region that contains two ORFs. One is called *vic*6 and the other is called *pix*6. Here the polymorphism is quite high for *vic* 6. *pix*6 has the whole internal region of the ORF totally conserved and the C-terminal region polymorphic.

Gil Choi, Institute for Bioscience and Biotechnology Research, University of Maryland, Shady Grove Campus

Vegetative incompatibility gene *vic6* and its non-allelic partner *pix6*. The *vic6* locus in EP155 is found on scaffold 3 in the JGI draft genome sequence assembly. The larger ORF in the predicted *vic6* locus was named *vic6* because it contained a HET domain which is frequently present in heterkaryon compatibility genes in *Neurospora* and *Podospora*. Among the 64 EU tester strains Michael Milgroom identified, EU21 differs from EP155 only at the *vic6* locus out of 6 loci. EU21 was the strain that was used to characterize the *vic6* locus. *C. parasitica* strain DK80, a derivative of EP155 and developed by Bao Chen in China, has a deletion of the *cpku80* gene. Consequently, it tends to have a higher frequency of homologous recombination (about 80%), and is a good strain to use in gene knockout experiments.

The gene *vic6* was disrupted in strains DK80 and EU21. With the disruption mutants, Choi used three different assays to see if the two mutant strains were compatible or not. First he tested vegetative compatibility. Pairing intact DK80 or EU21 against a mutant resulted in an incompatible reaction, but using two disruption mutants in which both *vic6* alleles are disrupted, a compatible reaction resulted, as shown in the table below.

Barrage Formation Test

Strain 1	Strain 2	Compatibility Reaction
DK80Δvic6#4	EU21 intact	Incompatible
DK80Δvic6#4	EU21Δvic6#63	Compatible
DK80 intact	EU21Δvic6#63	Incompatible

DK80Δvic6#4	EU21Δvic6#63	Compatible

The second test was virus transmission; if the strains are compatible, then there will be virus transmission without any hindrance. When both alleles of *vic6* were disrupted, there was 100% virus transmission. Interestingly, using EU21 strain as the virus donor and DK80 disruption mutant as the recipient, there was 100% transmission, even though, in the above table, EU21 intact was vegetatively incompatible with a DK80 disruption mutant.

***vic6* KO and virus transmission**

Virus Donor	Recipient	Conversion
DK80Δvic6	EU21	6/40 (15%)
DK80Δvic6	EU21Δvic6	20/20 (100%)
EU21	DK80Δvic6	30/30 (100%)
EU21Δvic6	EU21Δvic6	20/20 (100%)
EU21Δvic6	DK80	5/30 (17%)

Third, Myron Smith tested forced heterokaryon formation. Strains were developed that had hygromycin or neomycin markers. Strains were paired on PDA that contained both hygromycin and neomycin. The only way for the paired strain to grow is if a stable heterokaryon is formed which can express both antibiotic resistance markers. When DK80 is paired with DK80 derivatives, they all grow fine. When DK80 is paired with EU21, it does not grow. The same case occurs with EU21 and its derivatives. However, when the two derivatives are paired (DK80Δvic6 and EU21Δvic6), they grow well in the presence of two antibiotics, indicating that a stable heterokaryon has been formed. Data is shown in the table below.

	Mean Growth Rate Per Day (mm) of Paired Strains			
	Hygromycin Mutants			
Neomycin Mutants	**DK80**	**EU21**	**DK80Δvic6**	**DK80Δvic7**
DK80	+ (8.0)	- (<0.1)	+ (6.7)	+ (6.6)
EU21	- (<0.3)	+ (7.6)	- (<0.1)	- (<0.1)
EU21Δvic 6	- (<0.3)	+ (6.7)	+ (7.0)	- (0.2)

Three different assays employed showed that if the *vic6* allele is knocked out in two strains, they become compatible. This means that this gene *vic6* is involved in vegetative compatibility. Choi pointed out that there is directionality in virus transmission. Depending on which strain is the donor and which is the recipient, transmission rates will vary.

Choi tried *vic6* allele switching. When the allele 1 sequence was used to replace the allele 2 in DK 80, transformants on PDA did not grow well. For whatever reason, with the allele 1 sequence in the DK80 background, the fungus was not happy. There is some reason not to have allele 1 sequence in DK80. Myron Smith also was interested in the upstream ORF. Choi generated *pix6* mutants. (*pix6* is the smaller ORF upstream of *vic6*, **p**artner of vics**ix**). Null mutants were made, *EU21Δpix6* #15 and *EU21Δpix6* #17.

Barrage formation with *pix6* KO in DK80 & EU21

Strain 1	Strain 2	Vegetative Compatibility
DK80	EU21Δ*pix*6 #17	Incompatible
EU21Δ*vic*6	EU21Δ*pix* #17	Incompatible
EU21	EU21Δ*pix*6 #17	Compatible

DK80Δ*vic*6	EU21Δ*pix*6 #17	Compatible
DK80Δ*pix*6	EU21Δ*pix*6 #17	Compatible
DK80	EU21Δ*pix*6 #15	Incompatible
EU21Δ*vic*6	EU21Δ*pix*6 #15	Incompatible
EU21	EU21Δ*pix*6 #15	Compatible
DK80Δ*vic*6	EU21Δ*pix*6 #15	Incompatible
DK80Δ*pix*6	EU21Δ*pix*6 #15	Compatible

This particular gene does not contain any signature protein domains that are found in other HET (or vic) proteins. But, by knocking this gene out in both strains, the mutant strains become compatible indicating that this gene is involved in vegetative compatibility.

pix6 KO and virus transmission

Virus Donor	Recipient	Conversion
DK80Δ*pix*6	EU21	32/32 (100%)
DK80Δ*pix*6	EU21Δ*pix*6	20/20 (100%)
DK80Δ*pix*6	EU21Δ*vic*6	32/32 (100%)
EU21	DK80Δ*pix*6	0/30 (0%)
EU21Δ*pix*6	DK80Δ*pix*6	20/20 (100%)
EU21Δ*pix*6	DK80Δ*vic*6	20/20 (100%)
EU21Δ*pix*6	DK80	20/20 (100%)

There was a 100 % virus transmission when two mutant strains were used as donor or recipient. Again, there is asymmetric directionality in that EU21 as a recipient with DK80Δ*pix*6 as a donor yields 100% virus transmission, but there is no transmission when EU21 is the virus donor strain. Choi thought that by knocking out the *pix*6 gene, he could develop a universally efficient virus donor. To test this, he paired the null mutant against other EU tester strains, differing at *vic4*, *vic2* and *vic7* loci. The data is in the table below. There was 100% conversion with *vic4* (but this is idiomorphic and even without *pix6* disrupted, virus transmission is very efficient), but less efficient conversion with strains with different *vic2* and *vic7* loci.

Donor	Recipient	Conversion
DK80Δ*pix*6	EU1 (2212-22)	10/10 (100%)
DK80Δ*pix*6	EU6 (2111-22)	1/10 (10%)
DK80Δ*pix*6	EU18 (2211-21)	4/10 (40%)
EP 155 (2211-22)	EU1 (2212-22)	10/10 (100%)

It turns out the *pix6* disruptant is not going to be a universal virus donor. Choi then made a double knockout, knocking out both *pix6* and *vic6* genes in one strain. In pairing isolates for vegetative compatibility, the double knockout performed well as shown in the table below.

Barrage formation assay with _pix6/vic6_ double KO mutant of DK80

Strain 1	Strain 2	Compatibility
DK80Δ*vic*6Δ*pix*6	EU21Δ*pix*6	Compatible
DK80Δ*vic*6Δ*pix*6	EU21Δ*vic*6	Compatible
DK80Δ*vic*6Δ*pix*6	EU21	Compatible
DK80Δ*vic*6Δ*pix*6	DK80	Compatible

DK80	EU21	Incompatible

Choi then used the double knockout to test virus transmission. As a donor and recipient, there was 100% transmission both ways.

***vic6pix6* double KO and virus transmission**

Virus Donor	Recipient	Conversion
DK80Δ*vic*6Δ*pix*6	EU21	20/20 (100%)
EU21	DK80Δ*vic*6Δ*pix*6	20/20 (100%)

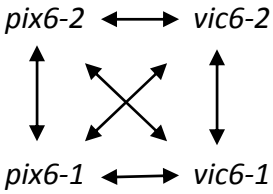
Choi presented a summary of his pairings, both for compatibility and for virus transmission. The table follows:

			VC Compatibility	Virus Movement	
Pairing	**Strain**	**Genotype**	**Barrage**	**DK80 to EU21**	**EU21 to DK80**
A	DK80 EU21	*pix6-2, vic6-2* *pix6-1, vic6-1*	Yes	5% (1/20)	15% (3/20)
B	DK80Δvic6 EU21	*pix6-2,* ***Δvic6-2*** *pix6-1, vic6-1*	Yes	15% (6/40)	100% (30/30)
C	DK80 EU21Δvic6	*pix6-2, vic6-2* *pix6-1,* ***Δvic6-1***	Yes	100% (20/20)	100% (20/20)
D	DK80Δvic6 EU21Δvic6	*pix6-2,* ***Δvic6-2*** *pix6-1,* ***Δvic6-1***	No	100% (20/20)	100% (20/20)
E	DK80Δpix6 EU21	***Δpix6-2****, vic6-2* *pix6-1, vic6-1*	Yes	100% (32/32)	0% (0/30)
F	DK80 EU21Δpix6	*pix6-2, vic6-2* ***Δpix6-1****, vic6-1*	Yes	7% (2/30)	100% (20/20)
G	DK80Δpix6 EU21Δpix6	***Δpix6-2****, vic6-2* ***Δpix6-1****, vic6-1*	No	100% (20/20)	100% (20/20)
H	DK80Δvic6 EU21Δpix6	*pix6-2,* ***Δvic6-2*** ***Δpix6-1****, vic6-1*	Yes	13% (4/30)	100% (20/20)
I	DK80Δpix6 EU21Δvic6	*pix6-2,* ***Δvic6-2*** *pix6-1,* ***Δvic6-1***	Yes	100% (32/32)	10% (3/30)
J	DK80Δpix6Δvic6 EU21	***Δpix6-2, Δvic6-2*** *pix6-1, vic6-1*	No	100% (20/20)	100% (20/20)

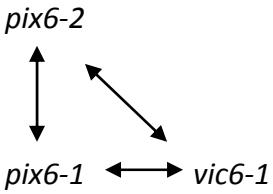
Of the ten pairings, three failed to produce a barrage line and those were when both alleles of *pic6* or *vic6* in two strains were disrupted or when both genes were knocked out in one strain. In those three pairings, virus transmission was 100% in both directions.

Choi showed the following diagram that represents two genes, two alleles in two different strains that differ at only the *vic6* locus. There are potential interactions between the two genes and two alleles. The vertical arrows represent allelic interaction. The slanted arrows

are non-allelic interactions. The horizontal arrows—there could be interactions within a strain between the two genes.



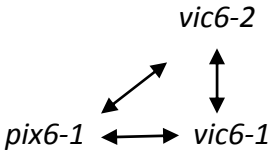
When *vic6-2* allele was knocked out, three interactions are knocked out, as show in the following diagram. Barrage line forms and there is virus transmission directionality.



When both *vic6* alleles are removed, there is only *pix6* allelic interaction left. But, that is not sufficient to kill those cells to stop virus movement from one strain to another, nor sufficient to provide vegetative incompatibility.

		Barrage	DK80 to EU21	EU21 to DK80
DK80Δ*vic*6 EU21Δ*vic*6	*pix*6-2, Δ*vic*6-2 *pix*6-1, Δ*vic*6-1	No	20/20 (100%)	20/20 (100%)

If one allele of *pix6* is knocked out (e.g. *pix6-2*), the other three interactions are intact. Barrage line still forms and there is directionality of virus movement.



		Barrage	DK80 to EU21	EU21 to DK80
DK80Δ*pix*6 EU21	*Δpix*6-2, *vic*6-2 *pix*6-1, *vic*6-1	Yes	32/32 (100%)	0/30 (0%)

When both alleles of *pix6* gene were knocked out, the only interaction left was allelic between *vic6* alleles and there is no barrage line and virus transmission is free. This interaction is not sufficient to provide incompatibility between the two strains.

vic6-2

vic6-1

		Barrage	DK80 to EU21	EU21 to DK80
DK80Δ*pix*6 EU21Δ*pix*6	*Δpix*6-2, *vic*6-2 Δ*pix*6-1, *vic*6-1	No	20/20 (100%)	20/20 (100%)

When there is a non-allelic interaction, it is sufficient to kill the cells so the barrage line is made and there is directionality of virus movement.

pix6-2

vic6-1

		Barrage	DK80 to EU21	EU21 to DK80
DK80Δ*vic*6 EU21Δ*pix*6	*pix*6-2, Δ*vic*6-2 Δ*pix*6-1, *vic*6-1	Yes	4/30 (13%)	20/20 (100%)

If two genes are knocked out in one strain, the remaining inter-genic interaction in one strain is not sufficient to form barrage line or to prevent virus transmission as shown below.

pic6-1 ⟷ *vic6-1*

		Barrage	DK80 to EU21	EU21 to DK80
DK80Δ*pix*6Δ*vic*6 EU21	*pix*6-2, Δ*vic*6-2 *pix*6-1, *vic*6-1	No	20/20 (100%)	20/20 (100%)

Therefore, non-allelic interactions are responsible to provide *vic6* locus-mediated vegetative incompatibility. The allelic interactions may still play a minor role, for instance the speed of cell death, the intensity of barrage line formation etc, but the major players in this vegetative incompatibility are the non-allelic interactions.

***vic* genes in *C. parasitica* field isolates.** The differences between EP155 and EP146 differ at vic loci 2, 4, 6 and 7 as shown in the table below.

***C. parasitica* isolate**	**EU Type**	***vic* genotype**
EP155	EU5	2211-22
EP146	EU17	2112-11

The target genes for PCR and sequencing are as follows below:

Locus	Gene
vic1	? (not confirmed)
vic2	*ptn, sec9*
vic3	?
vic4	*vic4* (idiomorphic)
vic5	--
vic6	*vic6, pix6*
vic7	*vic7*

Choi stated that when he checked EP155 and EP146 with certain sets of primers, he could see the difference in PCR product sizes to differentiate allele 1 from allele 2. When there was any ambiguity, the PCR products were used for sequencing. He was interested to see if allelic differences will hold up in field isolates. Field isolates that were tested were from: Depot Hill, NY; Finzel, MD; Bartow, WV; and, Crevoladossla, Italy.

A summary of data from the field isolates is shown in the table below.

vic gene alleles in American and Italian field isolates

Isolate	Previous Genotype	*vic*2(ptn)	*vic*2(sec9)	*vic*4	*pix*6	*vic*6	*vic*7	New Genotype
Ep 155	2211-22	2	2	1	2	2	2	
Ep 146	2112-11	1	1	2	1	1	1	
MD3	2112-11	1	1	2	1	1	1	
MD8	2212-12	2	2	2	1	1	2	
MD11	2212-22	1	1	1	1	1	1	2121-11
MD16	1212-22	2	2	2	2	2	2	
MD18	2211-11	2	2	1	1	1	1	
MD19	1122-11	1	1	2	1	1	1	
MD22	2112-12	1	1	2	1	1	2	
MD28	2212-21	2	2	2	1	1	1	1212-11
MD33	2211-22	2	2	1	2	2	2	
MD46	2122-11	1	1	2	1	1	1	
MD52	1211-12	2	2	1	1	1	2	
MD55	1112-21	1	1	2	2	2	1	
VO1	2112-22	1	1	2	2	2	2	
VO21	2112-21	1	1	1	2	2	2	2111-22
VO59	2111-11	1	1	1	1	1	1	
BRU2	2212-21	2	2	2	2	2	1	
BRU10	2222-12	2	2	2	1	1	2	
BRU16	2212-22	2	2	2	2	2	2	
BRU17	2112-21	1	1	2	2	2	1	
BRU19	2211-11	2	2	1	1	1	1	

BRU69	1211-11	2	2	1	1	1	1	
DU14	1212-11	2	2	2	1	1	1	
DU29	2212-22	2	2	2	2	2	2	
DU53	2122-21	2	2	2	2	2	2	X2x2-22
DU63	2111-11	1	1	1	1	1	1	
DU72	2122-12	1	1	1	1	1	1	2121-11
DU74	2211-12	2	2	1	1	1	2	

The association between *vic6* and *pix6* was strict. If one is allele 1, the other has to be allele 1. The same holds true for *ptn* and *sec9* as *vic2* genes

Asian isolates were included to expand the study, as Asian isolates should have had more time to evolve and may provide more diversity. Isolates were obtained from China (4 isolates), Japan (12 isolates) and Korea (10 isolates). Not many differences were seen.

vic gene alleles in Chinese and Japanese field isolates

Isolate	**ptn**	**sec9**	**vic4**	**pix6**	**vic6**	**vic7**	
Ep 155	2	2	1	2	2	2	2211-22
Ep 146	1	1	2	1	1	1	2112-11
9501	2	2	2	2	2	2	China
9502	2	2	2	1	1	1	
9503	1	1	2	1	1	2	
9504	2	2	2	2	2	2	
CD1	2	2	1	2	2	2	Kyoto
CD2	1	1	2	1	1	2	
CD3	2	2	2	2	2	1	
CD4	2	2	2	1	1	2	
JA1	2	2	1	1	1	1	Chiyoda
JA2	2	2	2	1	1	2	
JA3	2	2	1	2	2	1	
JA4	1	1	1	2	2	1	
JA19	2	2	1	2	2	1	Iwama
JA20	1	1	1	2	2	1	
JA21	2	2	2	2	2	2	
JA23	2	2	1	2	2	1	
JA60	2	2	1	2	2	2	Yasoto
JA62	2	2	1	1	1	1	
JA643	1	1	1	1	1	2	
JA64	1	1	1	2	2	1	
A1	1	1	2	1	1	2	Korea
B14	1	1	2	1	1	2	
EN211	2	2	2	1	1	1	
EW222	2	2	2	1	1	1	
FDS1	2	2	2	1	1	1	

JN322	2	2	1	1	1	2	
O131	2	2	2	1	1	1	
R112	2	2	2	2	2	2	
YS522	1	1	1	1	1	1	
Z5	2	2	2	1	1	2	

The association between *vic*6 and *pix*6 was the same. If one is allele 1, the other is allele 1 also. Again, the same holds true for *ptn* and *sec9*. There was no additional sequence variation seen among these Asian isolates except a few SNPs. With respect to more vc types reported among Asian isolates, Choi stated that, with this genotype information, it is now easier to test Asian isolates against the EU tester strains to determine if there are more vegetative incompatibility loci among Asian isolates as suggested by others.

Angus Dawe, New Mexico State University

Conserved and variable structural elements in the 5' UTR of *Cryphonectria* hypoviruses. Virulence-attenuating viruses (hypoviruses) of *C. parasitica*, have become a premier model for understanding the molecular biology of mycoviruses. However, a major gap exists in current understanding of structure and function of the untranslated regions (UTRs) of the hypovirus RNA genome, despite considerable evidence that secondary and tertiary UTR structure plays a crucial role in the control of translation and genome replication in other systems. In this study, a structure prediction software was used coupled with RNase digestion studies to develop validated structural models for the 5' UTRs of the two best- characterized members of the *Hypoviridae*, CHV1-EP713 and CHV1-Euro7. These two hypovirus strains exhibit significant variation in virulence attenuation despite sharing >90 % sequence identity. Their models reveal highly structured regions in the 5' UTR of both strains, with numerous stem-loops suggestive of internal ribosome entry sites. However, considerable differences in the size and complexity of structural elements exist between the two strains. These data will guide future, mutagenesis-based studies of the structural requirements for hypovirus genome replication and translation.

A role for the putative transcriptional regulator *Cpvib-1* in virulence, incompatibility-associated cell death and virus transfer. A gene has been identified within the *Cryphonectria parasitica* genome, *Cpvib-1*, that contains predicted protein domains similar to NDT80/PhoG and p53 transcription factor DNA-binding domains. This protein also shares significant similarity with VIB-1, a key regulator for several pathways leading to cell death in response to vegetative incompatibility in *Neurospora crassa*. By constructing a deletion strain lacking *Cpvib*-1, they report that this gene encodes a negative regulator of asexual sporulation and is also required for aerial hyphal growth on solid medium. Furthermore, the *ΔCpvib-1* strain was observed to be significantly impaired with respect to pathogenicity of live chestnut tissue. By using cell death counts following hyphal fusions of compatible and incompatible strains, they concluded that CpVib-1 is required for the progression of cell death and barrage formation in response to mismatches at *vic-4,* albeit in an asymmetrical manner. However, measurement of viral transfer indicated that there may be further involvement of CpVib-1 mediated signaling in response to signals propagated by mismatches at *vic-6*. Contrary to expectations, however, loss of *Cpvib-1* appears to further decrease the propensity for hypovirus transmission between incompatible

strains. This suggests that there are further complicating elements yet to be identified in this system.

Dawe's work in progress is as follows:

- To better define the relationship between Vib-1 and *vic* genes
    - Generate knockouts of Vib-1 in the background of other allelic variations besides *vic-4*
    - *Vic-6* and *7* contain HET-motif predictions, one idiomorph of *vic-4* has other related domains
- Identify targets and functional partners of Vib-1
    - Looks like a transcription factor
    - GFP labeling for localization, mutagenesis
    - CHIPSeq, transcriptomics → downstream targets
- Protein interactions
    - TAP Tag, Co-IP → upstream components

Ken Jensen, Institute for Bioscience and Biotechnology Research, University of Maryland, Shady Grove Campus

The mystery that is p48. The p48 protein is composed of 418 amino acids and is located at the N-terminus of open reading frame B (ORF B). The protease domain of p48, amino acid residues Cysteine 341 and Histidine 388 are responsible for the cleavage between p48 and ORF B. The protease domain of p48 resembles p29, a papain-like protease located at the N-terminus of ORF A, in terms of conserved amino acid sequences surrounding the catalytic cysteine and histidine residues. Unlike p29, p48 is not dispensable for viral RNA propagation. However, p48 can rescue the propagation deficiency when supplied in *trans*. Once rescued, the Δp48 mutant virus can continue to replicate in the apparent absence of p48. Thus, p48 has multiple functions, including an essential role in the initiation, but not the maintenance of hypovirus RNA propagation. However, Cys341, His388, and Gly418 can be mutated to Cys341Ser, His388Ser, Gly418Arg, individually or in combination and the virus will continue to replicate, and p48 cleavage still occurs. Mutations to the protease domain or the cleavage site result in a decrease in viral RNA and protein accumulation in EP155.

Jensen is furthering examining the essential functional domains of p48 by generating p48 deletion mutants. Six deletion mutants were generated starting at amino acid Threonine 14. Results indicate that the region corresponding to amino acids 14-330 can be deleted from p48, and CHV1-EP713 will still replicate. During construction of the deletion mutants, mutants Δ1 (amino acids 14-72), Δ2 (amino acids 14-124) and Δ5 (amino acids 14-273), contained a Serine in place of Threonine at amino acid 14, and either did not replicate (Δ1 and Δ5) or were deficient in viral RNA and protein accumulation (Δ2) when compared to the Δ3 (amino acids 14-168), Δ4 (amino acids 14-227), and Δ6 (amino acids 14-330) mutant viruses, that had Threonine at amino acid 14. Next he asked the question, 'is the amino acid at position 14 an essential position for the virus?' He mutated serine at position 14 to Threonine in the Δ1, Δ2 and Δ5 mutant viruses. Both the Δ1(T) and Δ2(T) mutant viruses, with Threonine at position 14 caused a much more debilitated fungal colony (similar to wild-type) than when serine was at position 14. What happens if he goes further into p48. Jensen began cutting into the protease

domain—he cut at Threonine 351, Threonine 374 and Threonine 394 and he has not found any replicating virus with these three mutants. All phenotypes look like EP155. He is in the process of repeating transfection with these mutant viruses. In summary,

- The protease domain of p48 can be mutated, and p48/ORF B cleavage will still occur.
- The p48 cleavage site can also be mutated individually or in combination with the protease domain and p48/ORF B cleavage will still occur.
- Deletion of amino acids 14-330 result in a decrease viral RNA and protein accumulation.
- Threonine at amino acid position 14 appears to be essential for virus replication.
- The p48 deletion mutants display a decrease in RNA and protein accumulation in EP155.
- The C-terminus of p48, amino acids 331-418 appear to be essential for viral replication.

Jensen is currently working on examining the any possible role of p29 in ORF B polyprotein processing and generating p48 C-terminus deletions.

Josh Springer, Michigan State University

Hypovirus inoculations in Michigan chestnut populations. Demographic models initiated by Anita Davelos Baines suggest that treating larger diameter chestnut trees is ineffective. They are attempting to protect American chestnut trees with diameters between 1 and 10 cm in three populations: Missaukee Healthy; Leelanau; and, Stivers. Trees were inoculated with hypovirus GH2 that was moved into the major vc groups at each population. Trees at these sites have been censused since 1996. Hypovirus inoculations were made yearly in 2009, 2010 and 2011. Inoculations in 2009 and 2010 were made by drilling holes into the stem and filling them with hypovirus slurry. However, the number of inoculation sites without visible stroma and the uptake of hypovirus into existing cankers were unacceptably low using this method. As a consequence, scratch inoculations were carried out in 2011. Dieback or smaller stems also has been high, thus in 2010 and 2011, inoculations included trees with diameters as large as 18 cm.

The following table indicates the number of dead trees at each of three sites from 2009.

Number of dead trees found in a two-year period at each site.

	Missauskee	Leelanau	Stivers
2009-2010	5	0	23
2010-2011	0	7	13

This year, 2011, was the final treatment year of hypovirus treatment. They will continue annual sampling and track the progress of treated trees. The annual census will continue and they will continue to compare existing recovering with inoculated trees.

Claire Moore, Michigan State University

Intra-canker variability for vegetative compatibility groups. Investigations aimed at determining the number of vegetative compatibility (vc) groups within an individual canker continue. The assumption is that most cankers harbor only one vegetative compatibility group.

This assumption may not be correct—what if there are multiple vc groups in a canker? This may pose implications for hypovirus treatment. In field studies, some cankers exhibit partial canker control—some areas of the canker are controlled by callus while another area of the canker may continue to grow unimpeded. Is this scenario caused by tree effect or might it be multiple vc groups in a canker? To test whether or not multiple vc groups exist within a canker, 30 cankers from the West Salem, WI chestnut site were examined. Six-to-twelve isolates per canker were paired among themselves. The results were that 22 cankers contained a single vc group while eight cankers contained 2 vc groups. Moore found that, in the case of cankers containing two vc groups, one isolate was found at a higher frequency (average of 84%) than the other isolate. Similar studies were initiated at four Michigan populations in 2011. Preliminary data for the Leelanau, Michigan population found that 68% (5/8 cankers) had two vc groups. Moore questions if the isolates within cankers with multiple vc group occur simultaneously or is one the primary infection and the other secondary? Are the isolates competing with one another in the canker? Moore will continue with these studies.

Sarah Bryner, WSL Swiss Federal Research Institute, Birmensdorf, Switzerland

Detecting and describing factors that govern the evolution of virulence in *Cryphonectria* hypovirus 1. In Europe, chestnut blight incidence is very high but the severity of the disease is low (due to *Cryphonectria* hypovirus 1).

***Cryphonectria hypovirus 1* (CHV-1)**

- dsRNA virus, infects fungus consistently
- Dispersed in asexual spores (conidia)—the virus needs a certain amount of sporulation for dissemination.
- Transmitted by hyphal anastomosis—induces a hypovirulent phenotype in *C. parasitica*: growth ↓, asexual sporulation ↓, no sexual reproduction, healing cankers.
- Biological control is successful in Europe—the virus is used very successfully in Europe as a biocontrol agent. It spreads naturally, but it was also helped by artificial introductions.
- Four subtypes of CHV-1 described:
  I (Italian), D (German/Spanish), F1 and F2 (French 1 and 2)

Project 1: Impact of temperature on the fungus-virus interaction (already published in American Naturalist, Jan 2011)

Climate chamber experiments:

- Fungus: 4 strains of *C. parasitica*
- Virus: 4 CHV-1 subtypes (I, D, F1 and F2) and no virus
- Temperature: 4 temperatures (12^{o}C, 18^{o}C, 24^{o}C and 30^{o}C)
- Growth and sporulation on agar medium were measured
- Growth on dormant chestnut stems was measured

Results were highly significant. The fungus, whether or not virus infected, grew more aggressively at higher temperatures. Temperature can alter the outcome of the fungus–virus interaction. There was a genotype by genotype by environment interaction. Some fungus/virus interactions grew best at 24 C, but much worse at 30C. As climate change occurs, this might mean that while some fungi are currently controlled by the virus, elevated temperatures may

break down that control and lead to more incidences of blight infections. Regions such as Turkey and Georgia have greater problems with chestnut blight and sending them hypovirulent isolates may not be practical because of these fungus/virus/environment interactions.

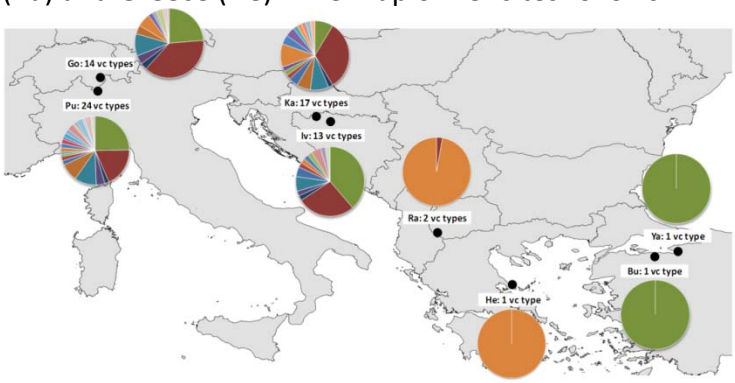
Project 2: Impact of vegetative incompatibility within *C. parasitica* populations on virulence of CHV-1

Evolution of parasite virulence theories:

- Trade-off hypothesis: Parasite needs to balance positive effects (benefits) of host exploitation on parasite multiplication within the host and the negative effects (costs) of this exploitation on transmission between hosts
- Trade-off in CHV-1 evident: High virulence reduces growth and sporulation of *C. parasitica* and, thus, the potential of CHV-1 to be transmitted to other hosts (high cost of virulence).
- Selection of low virulence in CHV-1 expected
- Transmission barrier: Vegetative incompatibility between fungal hosts further restricts virus transmission (programmed cell death after anastomosis)

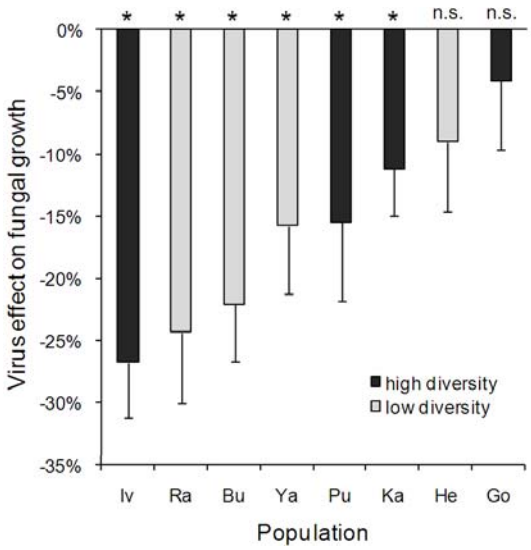
Therefore: Increased selective pressure towards low virulence of CHV-1 in *C. parasitica* populations with high degree of vegetative incompatibility (high diversity of vc types) is expected (biocontrol less effective).

To test if vc diversity affects CHV-1 virulence, eight populations of the fungus were chosen—areas with high vc diversity (Switz., Bosnia) and those with low vc diversity (Macedonia, Greece, Turkey). The CHV-1 subtype is found all across Europe from France and Italy to the Balkans and Turkey. The eight populations included: two populations each in Switzerland (Go, Pu), Bosnia (Ka, Iv) and Turkey (Bu, Ya) and one population each in Macedonia (Ra) and Greece (He). The map of her sites follows.



The vc types of all isolates were identified by testing with known vc type tester strains from Europe. Bryner used 16 virus-infected isolates from each population to see what their effect was on their host. She made all isolates virus free and grew the virus-infected and virus-free cultures on dormant excised chestnut stems and then looked at the difference using the following formula.

$$\text{Virus effect: } \frac{\text{(virus-infected fungus) – (virus-free fungus)}}{\text{(virus-free fungus)}}$$



Bryner's results were:

- Vegetative incompatibility of *C. parasitica* host population does not influence virulence of CHV-1.
- Most virulent viruses were found in a population (Iv) with high vegetative incompatibility.
- High variation of virulence within populations—she found viruses with low and high virulence in each population.
- CHV-1 was prevalent in all populations.

Bryner found no sign for evolution of avirulence of CHV-1 in *C. parasitica* populations with a high degree of vegetative incompatibility. Also, she found no sign for hindered transmission in populations with a high degree of vegetative incompatibility.

- Great promise for the sustainability of biocontrol system because the different degrees of vc diversity does not seem to be a barrier.
  - ⇨ Increase in vc type diversity (new introductions or sexual recombination) in Europe may not lead to erosion of biocontrol.
- Why can CHV-1 be highly virulent and still be transmitted – even when increased transmission barriers are present? Are there not only costs but also certain benefits of high virulence for transmission?

Project 3: Relationship between virulence and transmissibility in CHV-1

The common assumption is that virus transmission rate per host-to-host contact (transmissibility) is not influenced by virus strain – but this has never been tested.

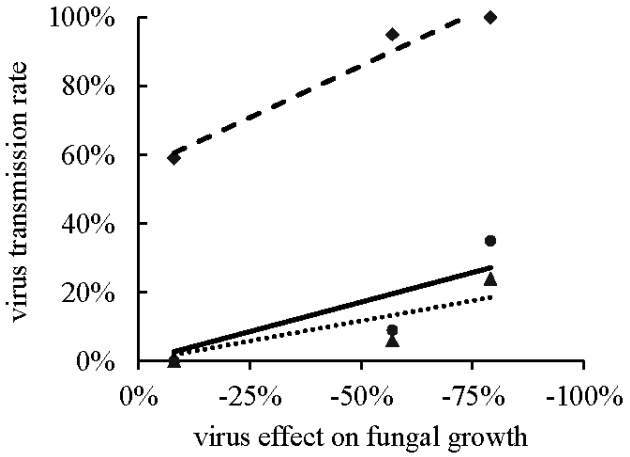
- More virulent viruses might be more transmissible.
- This virulence benefit would counterbalance the virulence cost on transmission, i.e. lower number of asexual fungal spores carrying the virus would be compensated by higher infectivity of these spores.

Bryner's transmission experiment was as follows:

- Transmission rate of three viruses with significantly different virulence was tested between different pairs of virus-infected fungal donor and virus-free fungal recipient strains.

- Three CHV-1 viruses with differing virulence were used (French subtype with high virulence, German subtype with intermediate virulence and an Italian subtype with low virulence).
- Two fungal donors—both had same vc type but different genotypes
- Eight fungal recipients (4 different vc types)
- Transmission was determined morphologically—100 replicates per virus and vc type combination were used.
- Virus strain has a significant impact on transmissibility between incompatible fungi (transmission between compatible fungi 100%).
- Virulence and transmissibility are positively correlated:

  High virulence is associated with increased transmissibility.



Virulence has not only costs (fungal growth & asexual sporulation ↓) but also benefits (infectivity of spores / virus transmissibility ↑) for transmission of CHV-1.

- Virulence may increase or decrease without selective disadvantages.
- …but only up to a certain threshold, above which dispersal is inhibited (not sufficient spores produced). The Italian subtype, which is quite mild in virulence, is widespread and has been persisting in populations for many years. Likewise, the German subtype also can be reisolated from populations were it has been previously detected. The situation is different with the French subtype. Robin and Rigling found in treatments done in France where very virulent forms were put out that 20 yearslater the French subtype could not be found anymore. However, when surveys are done in France and Spain, the French subtype is found repeatedly. It might be that the French subtype is at the threshold were some are too virulent to persist, while others are not.

Project 4: Population history of CHV-1 Italian subtype populations in Europe. The history of CHV-1 in Europe is not known.

- Most likely, the virus was introduced from Asia together with *C. parasitica.*
- Different CHV-1 subtypes may have been introduced independently.

Questions:

- What is the genetic relationship within and among CHV-1 Italian subtype populations?
- What about the relative age of different populations?
- Single or multiple MRCAs (most recent common ancestor)?

- Introduction with fungus?
  Sequencing of dsRNA: ~700 nt cDNA sequence (part of ORFA):
- 306 CHV-1 subtype samples from 8 European populations (2008/2010)
- several older CHV-1 subtype samples from Switzerland, Italy, Bosnia and Macedonia (1970 – 2000)

Bryner used coalescent analysis with BEAST software (BEAST: Bayesian evolutionary analysis by sampling trees). She presented preliminary data with very little data analysed. Of the samples that she tested, there is evidence that the Swiss populations are the oldest and those populations in Turkey are the youngest. Samples from Genoa, Italy (a point of origin for *C. parasitica* in Europe) were not part of her study.

William MacDonald, West Virginia University

Introduction of hypoviruses at West Salem, Wisconsin (in cooperation with J. Cummings-Carlson, Wisconsin Department of Natural Resources; D.F. Fulbright and A.M. Jarosz, Michigan State University; and, M. Milgroom, Cornell University). The stand of American chestnut in West Salem became infected with chestnut blight in the late 1980s after 100 years of blight-free growth. Hypovirus introduction (individual canker treatment) was conducted from 1992-1997 (700 cankers on 133 trees received inoculum). From 1998-2002 hypovirus introduction was halted. In 2001, due to a large increase in the number of cankers in the stand, twelve permanent plots were established in three regions of the stand representing differing levels of disease: Disease Center; Front; and, Beyond the Front. Hypoviruses were reintroduced in 2003; annual treatment has continued through 2011. Approximately 25% of the trees in each plot are untreated to assess tree-to-tree spread of hypovirulent strains.

Hypovirus spread has been assessed annually by analyzing isolates of *C. parasitica* that arise from bark samples. Hypovirulent isolates are recovered most readily from treated cankers followed by non-treated cankers on treated trees. Hypoviruses have spread less effectively to untreated trees. Since 1992, a total of 3040 cankers have been identified in the 12 plots. Four-hundred, fifty-six cankers on living trees were sampled in June 2011; 159 were newly discovered.

General observations:

- Disease Center Plots—over 90% of the trees are infected. However, the disease in this area appears to have stabilized. Most cankers (75%) are hypovirulent in appearance with little evidence of stroma. Fifty-seven percent of the trees that initially were treated with hypoviruses from 1992-1997 were alive in 2011. In contrast, only 40% of the trees infected between 1998-2002, in the absence of hypovirus treatment, remain alive.
- Disease Front Plots—Disease incidence has increased from 29% in 2001 to 95% in 2011. Recovery of hypovirulent isolates continues to increase. The number of cankers yielding hypovirulent isolates rose from 35% in 2005 to 64% in 2011.
- Beyond the Front Plots—Disease incidence has intensified since 2001, from 11% in 2001 to 91% in 2011. Recovery of hypovirulent isolates is lowest in these plots. The percent of cankers harboring hypovirus has remained almost unchanged since 2001 (~35%).

- Vegetative compatibility—WS-1 continues to be the dominant vc type in the stand; its frequency has declined in 2011 to 77%. WS-2 and WS-3 were found at rates of 2% and 16%, respectively.

The biological control potential of *Cryphonectria parasitica* strains containing an infectious cDNA copy of the hypovirus CHV1-Euro7 (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 cytoplasmically-infected counterparts. Three treatments compared transgenic hypovirulent (TG), cytoplasmic hypovirulent (CH), and virulent (V) strains. To produce ascospore inoculum, cankers were spermatized by painting cankers three times each summer (2004-2009) with a conidial mixture containing MAT-1 and MAT-2 mating types 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 177 natural cankers in TG plots, 113 in CH plots, and 105 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.

Significant findings this past year include the increased detection of hypoviruses, both in the canker thallus and in perithecial outcrosses. Beginning in 2007 and continuing through 2010, hypoviruses have been detected in cankers on trap trees, both in the thallus and in ascospores. Transgenic inoculum has been detected not only in TG plots but also in CH and V plots. 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%). The trees were not treated or examined in 2011; however, bark plugs were taken in Nov 2011 to see if outcrossing can still occur despite a lack of treatment in 2011.

Evaluation of Select Chestnut Sites in the Great Smoky Mountains National Park for Putatively Hypovirulent Isolates of *Cryphonectria parasitica.* (in conjunction with Glenn Taylor, Great Smoky Mountains National Park, Gatlinburg, TN). Populations of American chestnut trees in the Great Smoky Mountain National Park were examined in the fall of 2010 for the presence of infections caused by *C. parasitica*. Of the 852 bark plugs processed, 771 yielded *C. parasitica* isolates (90%). Only four isolates (all from one tree) were hypovirulent; the other 767 *C. parasitica* isolates appeared to be virus-free based on their culture morphology. The remaining 36 isolates were other bark organisms (*Trichoderma* sp., *Penicillium* sp. and *Pestalotia* sp.). The one putative hypovirulent isolate was single spored (50 individual asexual spores) and ten of the single spore colonies (20%) resembled known hypovirus-containing isolates. When dsRNA was extracted, the isolates each contained a single band of dsRNA.

All *C. parasitica* isolates were paired individually on a bromcresol green medium with the 64 European Union isolates. Forty-eight EU compatibility types were found multiple times and 22 vegetative compatibility groups were identified by a single isolate. One isolate was incompatible with all 64 EU testers. These data indicate a high level of diversity exists among

the *C. parasitica* population, suggesting that there may be significant genetic restriction to hypovirus transmission.

Additional bark samples at four different sites were collected in October 2011 and will be evaluated similarly to those collected in 2010.

Evaluation of *Cryphonectria parasitica* from a chestnut plantation on Bunner's Ridge, Marion County, WV. M. Haggblade (Pomona College undergraduate student summer intern). Approximately 400 chestnut trees from various sources (Michigan, irradiated and a few other sources) were planted (1988-1990) in a cleared area near Morgantown, WV. In 1998, a student from France (Noémie Biegbeder) sampled approximately 25 virulent cankers at the site that had developed naturally. Biegbeder, using some of David Huber's isolates, converted many of the virulent isolates and reintroduced them into the cankers from which they arose. The cankers at the site remained undisturbed since the experimentation by Beigbeder. Tree survival and cankers with unusual morphology prompted further study at the site.

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

Eric Goddard, West Virginia University

Assessment of the saprophytic growth of virulent and hypovirulent *C. parasitica* on stacks of dead American chestnut (*Castanea dentata*) and scarlet oak (*Quercus coccinea*). The saprophytic growth of *C. parasitica* is being studied to measure the potential of hypovirulent strains to produce higher levels of hypovirulent inoculum when grown saprophytically rather than in living stems. American chestnut and scarlet oak stems were cut in May, labeled and placed in replicate stacks in an understory setting. Sets of cut stems were inoculated immediately upon cutting and at 6-8 week intervals after cutting with either virulent, hypovirulent isolates or water agar. Canker growth and sporulation (subjective 0-3 scale) have been measured at monthly intervals. Pycnidia collected from bark samples will be used to measure the production of hypovirulent and virulent conidia. Other saprophytes that colonize the stems will be identified and their relationship to colonization by *C. parasitica* determined.

Lillibeth Northern, SUNY-ESF

American chestnut research and restoration project. Northern gave an overview of the work being conducted at SUNY-ESF. With regard to the chestnut restoration project, more than 50 researchers have contributed to the project over the last twenty years.

The following vectors were all cloned by Kathleen Baier who then sent them to Scott Merkle's lab in Georgia. Merkle inserts genes into FHI binary vectors and sends them back to SUNY-ESF.

Chestnut blight resistance candidate genes (CGs) cloned from *Castanea mollissima* and placed in binary vectors

	***C. mollissima* CG, putative ID**		***C. mollissima* CG, putative ID**
1	β-1,3 glucanase	12	Shikimate dehydrogenase
2	CBS domain protein	13	Myo-inositol-1-phosphate synthase
3	UDP glucosytransferase	14	Triacylglycerol lipase
4	Thaumatin-like protein	15	ACC oxidase
5	DAHP synthase (DHS1)	16	Cinnamy-alcohol dehydrogenase
6	Acid phosphatase	17	Peroxidase
7	Laccase/diphenol oxidase	18	CCoAOMY (Caffeoyl-CoA-O-methyltransferase
8	Proline-rich protein	19	Glucanase (glucan endo-1,3-glucosidase
9	Ethylene-response transcription factor	20	GST U7 (glutathione S transferase)
10	Cysteine proteinase inhibitor	21	Glucanase (glucan endo-1,3-glucosidase
11	Lipid transfer protein SSH	22	Germin-like protein

Phytophthora resistance candidate genes (CGs) cloned from *Castanea mollissima* and placed in binary vectors

	Chinese chestnut CG, putative ID
1	RPH1 (*Phytophthora* resistance)
2	NPR3/4 (*Phytophthora* resistance)

To date:

- Genes cloned
  - 21 cloned from Chinese chestnut
  - 4 non-Chinese genes
- Vector constructs
  - 23 single resistance-enhancing gene constructs
  - 3 pyramid (multi-gene) constructs
- Confirmed events in the pipeline
  - 115 events (using 12 vector constructs)
    - Only 1 , 2, or 3 events will move toward restoration program
- Initial resistance assays (small stem field assays)
  - 3 events
    - Wirsig (with deletion in promoter, previously tested)
    - Darling4 and Darling5 (small stem assay tested this summer)

Small stem assays. This is a test for high levels of resistance (not intermediate). Northern showed photographs of a 4-year-old tree, 1 1/2 inches (3.8cm) in diameter that was inoculated with *C. parasitica* to evaluate the level of resistance. In comparison, she also showed photographs of a 2-year-old tree ('Darling4'), 0.4-0.6 inches (1 to 1.5cm) inoculated with Ep 155 and SG 2-3 isolates. While 'Darling4' is slow to girdle, it is not as resistant as Chinese chestnut.

There is a new wave of trees to test:

- >400 events planned between SUNY-ESF and the University of Georgia

    - 31+ vector contructs
- 115 events in the pipeline
    - 112 vector contructs
- Tested two 'Darling' events this summer

Four-hundred+ events x 10 to 20 trees each = 4,000 to 8,000 or more trees to test.

Flowering chestnuts. In 2011, controlled crosses were attempted for the first time:

- Male flowers on a transgenic, and female flowers on several non-transgenics.
- Nuts will be planted this spring.
- Then they will test if the transgenes are in the seedling progeny.
- They also will test if the seedling transgenics grow as rapidly as non-transgenic seedlings.
- Polishing the pipeline—a shade house was built for acclimatization.

Debora Jacob-Wilk and Pam Kazmierczak, UC Davis (submitted report).

Kex-2 processing. Kex2-silenced strains of Cryphonectria parasitica, the ascomycete causal agent of chestnut blight, show a significant reduction in virulence, reduced sexual and asexual sporulation and reductions in mating and fertility. Due to this and the known involvement of Kex2 in the processing of important proproteins in other systems, we searched the whole *C. parasitica* genome for putative Kex2 substrates. Out of 1,299 open reading frames (ORFs) predicted to be secreted, 222 ORFs were identified as potential Kex2 substrates by this screen. Within the putative substrates we identified cell wall modifying proteins, putative proteinases, lipases, esterases and oxidoreductases. This in silico screen also uncovered a family of nine secreted aspartic proteinases (SAPs) of *C. parasitica*.

Northern blot analyses of this gene family showed differential expression when exposed to chestnut wood and Cryphonectria hypovirus 1 (CHV1). Due to the reduction in fungal virulence known to be caused upon hypoviral infection of C. parasitica, the differential gene expression observed, and the known involvement of SAPs in virulence in other systems, we conducted deletion analyses of four of these proteinases, representing different expression patterns. Deletion of each of the four SAPs did not affect growth rates, sporulation or virulence, suggesting that none of the considered SAPs is essential for the full development or virulence of *C. parasitica u*nder the conditions tested.

Results showing that proteases are the second biggest group of secreted-putative Kex2 substrates, and the fact that SAPs are differentially regulated upon exposure to chestnut wood are suggestive of the importance of proteinases at the fungal-host interface, yet their exact function remains elusive.

Cryparin study. Infection of the chestnut blight fungus *Cryphonectria parasitica* with the *Cryphonectria hypovirus 1* (CHV1) causes disruption of virulence, pigmentation and sporulation. Transcriptional down-regulation of key developmentally regulated fungal genes occurs during infection but vegetative growth is unaffected. Previous studies showed that CHV1 utilizes *trans*-Golgi network (TGN) secretory vesicles for replication. In this study, the fungal cell-surface hydrophobin cryparin was chosen as a marker to follow secretion in viral-infected and non-infected strains. Subcellular fractionation, cryparin-green fluorescent protein (GFP) fusion and Western blot studies confir med that vesicles containing cryparin co-purify with the same fractions previously shown to contain elements of the viral replication complex

and the TGN resident endoprotease Kex2. This vesicle fraction accumulated to a much greater concentration in the CHV1 infected strains than in non-infected strains. Pulse chase analysis showed that the rates and amount of cryparin being secreted by the CHV1 containing strains was much lower than in non-infected strains and the dwell time of cryparin within the cell after labeling was significantly greater in the CHV1-infected strains than in the non-infected ones. These results suggest that the virus perturbs a specific late TGN secretory pathway resulting in buildup of a key protein important for fungal development.

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.

Steven Jakobi, Alfred State College

Crowding study. Jakobi has been at Alfred State College in western NY since 1993. Alfred State has a full agriculture department, but no research activities. After the 2007 NE-1033 meeting, Jakobi contacted Sara Fitzsimmons for ideas for a small research project. Fitzsimmons offered several suggestions and they eventually agreed on a crowding study. The study, set on part of a 350-acre farm at Alfred State College, mostly dairy operations, was started in May 2008. Jakobi's study is a 0.2 acre plot, on a 5% slope. The area was cleared; Roundup® was then used to kill vegetation prior to planting. Fitzsimmons provided nuts (American, Chinese (2 sources), backcross hybrids—B_2F_3 and B_3F_2). Jakobi had a large number of people helping to plant 375 nuts. Blue plastic tree tubes were used as was a seven-foot deer fence. The soil is heavy clay with a pH of 5.8. Holes were dug, backfilled with good soil and fertilizer was added. Trees were planted either one, two or three feet apart to examine the effect of spacing on growth. Distance between rows was eight feet to allow for mowing. Border trees were planted using B_3F_1 nuts. After the nuts were planted, the site was not visited for more than a month, and at that time, the weeds were taller than the tree tubes. Since then, Jakobi has been mowing regularly.

Germination success and survival of different chestnut species and cultivars after 3 months in August, 2008.

Species/cultivar	Percent nuts germinated	Percent seedlings dead
Chinese (Entriken)	47/60 (78.4 %)	1/60 (1.7 %)
Chinese (Merkle)	33/60 (55.0 %)	--
American	30/60 (50.0 %)	2/60 (3.4 %)
B2F3	38/60 (63.3 %)	5/60 (8.3 %)
B3F2	43/60 (71.6 %)	5/60 (8.3 %)
Border (B3F1)	52/75 (69.3 %)	5/75 (6.7 %

Percent chestnut seeds/seedlings failed to germinate/dead at the ASC college farm site over a 3-year period. The initial planting consisted of 375 nuts in May, 2008; re-plantings occurred in May 2009 and 2010.

2009		2010		2011	
May	Aug	May	Aug	May	Aug

Chinese 1	25.0	11.7	16.7	15.0	20.0	18.3
Chinese 2	51.7	21.7	28.3	16.7	28.3	28.3
American	60.0	13.3	23.3	26.7	35.0	36.7
B2F3	51.7	16.7	26.7	13.3	20.0	26.7
B3F2	43.3	21.7	28.3	23.3	40.0	40.0

Voids were replanted in 2009 and 2010, but not in 2011.

Chestnut blight was noticed first in 2011. Jakobi presumes that the pathogen is seed-borne. Loss of trees due to chestnut blight may preclude any meaningful results. In addition, there is clearly some mortality by a canker disease other than *Cryphonectria*. Also evident is damage by rodents and Japanese beetles.

Mark Coggeshall, University of Missouri

Coggeshall works for the University of Missouri Agroforestry Center and they are trying to utilize chestnut as a specialty crop in Missouri.

Genetic Resource Information Network-GRIN (USDA-ARS network). The UM Agroforestry Center is beginning to load some of their phenotypic data onto this website.

A listing of accessions at the Horticulture and Agroforestry Research Center (HARC), an area about 25 miles west of Columbia, MO are listed below.

Listing of *C. mollissima* accession at the Horticulture Agroforestry Research Center (HARC)

Amy	Crane	Lindstrom #43	Norman 65
Armstong	Douglas #1A	Little Giant	OK Kwang
Auburn-Homestead	Everfresh	Meiling	Orrin
Auburn-Cropper	Ford's Sweet	Miller 72-105	Payne (Byron 3-3)
Auburn-Leader	Ford's Tall	Miller 72-76	Peach
Auburn-Super	Gideon	Mossbarger	Perry (Lindstrom 93)
Benton Harbor	Hemming	NC-8	Qing
Beth	Hong Kong	Norman Dwarf	Shing
Byron	Jersey Gem	Norman H2	Stark's Choice
Byron (Lindstrom 67)	Kintzel	Norman J160	Yixin
Campbell NC-8	Kohr	Norman J26	
Carr	Lee Wheeping	Norman J29	

Listing of *Castanea* interspecific hybrids at HARC

Accession	Cultivar	Pedigree	Accession	Cultivar	Pedigree
MACS 8	Basalta #3	sat. x cren.	MCAS 42	Marigoule	cren. X sat.
MACS 12	Bost	mixed	MCAS 47	Marsol	cren. X sat.
MACS 13	Bouche de Betizac	sat. x cren.	MCAS 52	Paragon	sat. x cren.
MACS 17	Carolina	moll. x dent.	MCAS 66	Precoce Migoule	cren. x sat.

MACS 19	Colossal	cren. x sat.	MCAS 67	Primato	cren. x sat.
MACS 21	Dallas-River	cren. x dent.	MCAS 69	Revival	moll. x sat.
MACS 22	Dallas-Street	cren. x dent.	MCAS 71	Simpson	moll. x sat.
MACS 24	Eaton	moll. x (cren. x dent.)	MCAS 72	Sleeping Giant	moll. x (cren. x dent.)
MACS 34	Layeroka	sat. x cren.	MCAS 74	Willamette	moll. x dent.
MACS 36	Linden seedling	cren. x sat.	MCAS 77	Yolo Grande	cren. x sat.
MCAS 40	Luvall Monster	cren. x	MCAS 78	316 #149	moll. x (cren. x dent.)
MCAS 41	Maraval	cren. x sat.	MCAS 79	416 #150	same

The information entered in the GRIN system was keyed as MCAS (Missouri *Castanea*). Almost all of the plants above were propagated by Ken Hunt at UM; Hunt has retired and his position may not be filled. Hunt has been collecting the following descriptor data since 2002:

- Leafing date
- First male/female flower
- Last male/female flower
- Secondary fruiting
- Nut yield per tree
- Nut size
- Nut maturity date
- Stem diameter (DBH)

Descriptor data for 79 *Castanea* accessions were uploaded to the GRIN database by Michele Warmund and Ken Hunt in fall 2010. They worked in collaboration with Kim Hummer, ARS curator/research leader at NCGR in Corvallis, OR.

Dennis Fulbright, Michigan State University

County Line. The County Line recovering American chestnut site was purchased in the summer of 2011 by Michigan State University for the purpose of continued studies on hypovirulence and the natural recovery of the American chestnut at that site. Andrew Jarosz is now in charge of the County Line site.

Michigan chestnut industry. Fulbright's four areas to help develop the chestnut industry in Michigan include:

- Germplasm and site selection—his concern is that many chestnut orchards in MI were set up with seedlings, in get-rich-quick schemes. Good quality germplasm is a necessity in good agricultural practices. 'Nevada' (a Japanese x European hybrid) has failed in many areas of MI because the climate is too cold. Thus, other cultivars are needed to replace 'Nevada'. 'Precoce Migoule' (Japanese x European hybrid) is a good alternative pollinizer. 'Benton Harbor' (grafted selection from Chinese chestnut seedling) originally planted in 1992 at the Benton Harbor horticulture research station is a very vigorous tree that produces a lot of flowers and pollen. It grafts well and can survive on many different sites and also resists frost. 'Labor Day' comes

from the late Norman Higgins collection and is thought to be of Korean origin. It has early maturity, important in northern Michigan.

- Horticultural care
- Harvest and storage—after harvest, chestnuts become highly contaminated with mold, yeast, bacteria. Treatments to evaluate antimicrobial products showed that StorOx® (hydrogen peroxide, at a concentration higher than available in stores) works well and consistently maintained quality. This product is EPA approved but not EPA regulated.
- Product development and marketing

Chestnut internal kernel breakdown (IKB). Little is known about internal breakdown other than it can appear where Japanese and Japanese X European hybrids are planted. In Michigan, it appears as a decaying kernel in otherwise healthy-appearing chestnuts. Experienced growers have suggested that they have seen it only in chestnut hybrids including hybrids of Japanese, European and American. No reports exist of kernel breakdown appearing in pure Chinese or pure European species. Pollen influence or a maternal segregation issue is suggested as it commonly appears in only one nut per bur (as opposed to all three). If a nutrient deficiency, all nuts in the bur and most nuts on the tree would show symptoms. Only 10-30 percent of the nuts show kernel breakdown on a tree. Sometimes double embryos within a nutshell are found but only one of the two embryos is afflicted within a single nut. The possibility that it is caused by Chinese pollen was a hypothesis; however, it has been observed on chestnuts where Chinese pollen could not have been the pollen source. In Michigan, most kernel breakdown has been effectively observed when 'Colossal' is the mother tree; however, kernel breakdown has not been effectively monitored in other French hybrids. If decay is found in Chinese chestnut, it has always been caused by a culturable microorganism. There are orchards of 'Colossal' trees that have never shown kernel breakdown leading to speculation that an infectious agent is involved. After several years of attempting to culture fungi, bacteria and yeast, no organism has been cultured from nuts showing kernel breakdown. The overall objective of this study was to determine the cause of internal kernel breakdown in 'Colossal' chestnut. To investigate this issue, crosses were set up between various pollen sources and the cultivar 'Colossal'.

'Colossal' pollinized by:	**Site 1 (4 Trees) IKB/nuts/flowers**	**Site 2 (4 Trees) IKB/nuts/flowers**	**Site 3 (6 Trees) IKB/nuts/flowers**
'Nevada'	0/119 (171)	0/97 (90)	0/189 (245)
'Okei'	-	2/189 (237)	-
'Precoce Migoule'	0/47 (204)	-	-
'Benton Harbor'	-	39/106 (106)	74/237 (567)
No pollen	0/0 (106)	0/2* (88)	5/11* (298)
	No pollen in air prior to bagging	*Possible 'Okie' pollen in air prior to bagging flowers	*Possible 'Benton Harbor' pollen in air prior to bagging

'Colossal' = *C. crenata* x *C. sativa* hybrid; 'Nevada' = *C. crenata* x *C. sativa* hybrid; 'Okei' – *C. crenata* x Chinquapin hybrid; 'Precoce Migoule' = *C. crenata* x *C. sativa* hybrid; 'Benton Harbor' = grafted selection from a *C. mollissima* seedling.

These first-year data suggest that Chinese chestnut, represented by 'Benton Harbor' (30%) and 'Okei' chestnut are somehow responsible for the appearance of IKB in Michigan 'Colossal' chestnut, and that 'Nevada' and 'Precoce Migoule' do not appear to cause it.

Antimicrobial activity of chestnut pellicle and shell extracts. Chestnut extracts were studied for antimicrobial activity against microorganisms, including plant pathogens. Chestnut extract on paper discs was applied to an agar medium to evaluate the inhibition to multiple microorganisms, or the extract was added at various concentrations to a culture medium to evaluate the growth of target microorganisms. Chestnut type, tissue of plants (shell, pellicle and leaf), extraction methods and physical characteristics were studied to determine antimicrobial activity. Most test microorganisms were inhibited by the extracts at different effective concentrations for 50% growth inhibition (EC50). *Pseudomonas fluorescens* was the most sensitive (EC50=4.4 µg/µl). *Phytophthora cambivora* (isolated from chestnut in Indiana) was one of the least inhibited (EC50=185 µg/µl) and *Cryphonectria parasitica* was not inhibited. Extracts of the Japanese x European chestnut (*C. crenata* x *C. sativa*) cultivar 'Colossal' showed a greater inhibition than those of wild trees of the Chinese chestnut species (*C. mollissima*). High temperatures did not affect the inhibitory effect. Extracts from chestnut pellicle had the highest concentration of antimicrobial compound, compared with leaf and shell. The active fraction contained several substances with molecular masses consistent with one flavonol glycoside and several terpenoid substances. Pellicle and shell tissue reduced radish scab disease caused by *Streptomyces scabies* in the greenhouse.

Lynne Rieske-Kinney, University of Kentucky

Asian chestnut gall wasp (ACGW) update. *Dryocosmus kuriphilus*, the Asian chestnut gall wasp, is rapidly expanding its range in North America. *D. kuriphilus* produces one generation per year; it is very small and it lays its eggs in the chestnut buds. The eggs hatch, grow very slowly initially and then spend the winter as very small larvae within the bud. Essentially, the insect is invisible to the naked eye and the only way to tell if a bud is infested or not is to dissect it. When the buds start growing in the spring, rapid gall formation is induced and the larvae develop within those galls. Galling will have a variety of impacts on the plant. Galls prevent:

- normal shoot growth and development
- flowering
- fruit set
- nut production

Tree mortality can occur in extreme cases.

Rieske-Kinney spoke on two research objectives. The first objective was to characterize the natural enemy complex of ACGW, including natural enemy recruitment and ecological associations. After ACGW was released in the U.S. in the 1970s, natural enemies were introduced about three years later. One of the introduced parasitic insects was *Torymus sinensis*. There are no good records as to exactly what parasitoids were released.

Season	Summer	Fall	Winter	Spring
ACGW	Adults emerge and lay eggs;	Larvae dormant	Larvae dormant	Larvae feeding, galls expand

	eggs hatch			
Torymus sinenis	Larvae feed on ACGW	Pupa	Pupa dormant in gall	Adults lay eggs; larvae feed on ACGW

T. sinensis has one generation per year, as does ACGW. It is perfectly synchronized to effectively parasitize ACGW; it can parasitize other gall wasps. Rieske-Kinney reported that *T. sinensis* is expanding its range in N. America coincidentally with ACGW.

O. labotus is a native parasitoid that produces several generations per year.

- The first generation is synchronized with ACGW and the second generation parasitizes *T. sinensis*; this is hyperparasitism. There is an antagonistic relationship developing between the introduced and native parasitoids. This may compromise the efficacy of the introduced parasite in regulating gall wasp populations.
- To make the situation more complex, three additional parasitoids have been discovered. They have not been identified, but one is tentatively a Braconid, a little unusual for this type of situation. They do not know what the relationships are—it is unknown if these new parasitoids are parasitizing the ACGW, *T. sinensis or O. labotus*. It is a very murky picture at this point.

The second objective was to evaluate interactions between a stem-cankering fungal pathogen and the gall wasp, which is an obligate plant parasite. Gall wasp infested seedlings were infected with a naturally occurring *Nectria* species stem canker. Graduate student, Ignazio Graziosi put together a 2 x 2 factorial experiment with: (1) fungus—canker and no canker (*Nectria*); and, (2) ACGW—gall or no gall. Seedlings that had both cankers and galls grew significantly less than the controls or just blighted seedlings. Also measured was the number of basal sprouts and only those seedlings with cankers produced basal sprouts. In looking at the fitness of the galls, Graziosi found no effects of the fungus on the gall wasp. Seedlings with cankers and galls had significantly less healing area than seedling with only cankers, suggesting the presence of the gall wasp impedes the seedling's ability to defend itself. In summary, seedlings with both cankers and galls tend to grow less than seedlings with only one or neither. The presence of the fungus stimulates the production of basal sprouts. The fungus had no effect of the gall wasp and the gall wasp compromised the ability of the seedlings to produce healing callus tissue, affecting fungal activity. The interactions between the ACGW and a stem cankering fungus have a negative impact on plant fitness, and a positive impact on fungal fitness.

Sandra Anaganostakis, Connecticut Agricultural Experiment Station

Gall wasp. Asian chestnut gall wasp was discovered on one tree at Lockwood farm in Hamden, CT in June 2011. Samples were sent to Lynne Rieske-Kinney for confirmation. This tree is the farthest south on the property, and is the first discovery of this pest in Connecticut. Other chestnuts north of this tree had no galls this year.

Phytophthora. Chestnut trees planted by Stacy Clark (USDA Forest Service) in TN and NC have had very high mortality because of root rot which appeared to be ink disease. Isolates from many of these trees were white and fluffy on PDA and appeared to be *Phytophthora*, but

kit tests were negative. The last isolates were grown in continuous light, and sporulated and were identified as *Phymatotrichopsis omnivora* which causes a root rot of cotton and many other species. It has been reported as a pathogen of chestnut in Texas. This Texas root rot had not been previously reported from TN or NC.

Seedling production systems (in conjunction with Scott Schlarbaum, University of Tennessee). In the fall of 2010, 8,000 nuts were collected from several crosses and open pollinated trees. Half (by weight) were taken to Forrest Keeling nursery in Missouri for planting in modified root production method (RPM™)containerization system and half went with Schlarbaum for planting at the East Tennessee State Nursery for production of bare-root seedlings. The resulting seedlings from both production systems will be planted in pairs in the spring of 2012 at: the Milford Experimental Forest (extreme northeastern PA); Delaware State Forests (northeastern PA); United State Military Academy (NY, above the Hudson River); Goshen, CT (in the northwestern corner of the state); Southington, CT (in the center of the state); and, at Griswold, CT (in the southeastern corner of the state). These planting will allow them to compare the efficiency of two seedling production methods with a variety of chestnut genotypes in a diversity of soil types and climates.

Scott Schlarbaum, University of Tennessee (submitted report)

The University of Tennessee's Tree Improvement Program (UT-TIP) cooperated with Dr. Stacy Clark, USDA-Forest Service, Southern Research Station (lead scientist) and regional geneticist, Barbara Crane, USDA-Forest Service, Southern Region, in establishing four additional chestnut plantings using material supplied by Dr. Fred Hebard, TACF®. The plantings contained different hybrid breeding generations, including BC_3F_3 generation, along with American and Chinese seedlings and were established on southern National Forests at undisclosed locations to prevent theft. Each planting site consisted of approximately 300 trees, or 600 trees planted on each forest, with 300 seedlings on midstory removal sites and 300 seedlings on shelterwood harvest sites.

Chestnut sawfly was discovered on a chestnut planting in Kentucky in 2009. Greater predation damage was found on seedlings in sites with lower harvest intensity than those in sites with greater harvest intensity. Chinese chestnuts suffered less predation than did American and BC chestnut seedlings. This defoliating insect has only been observed several times in the last century.

Annual measurements were made on a small plantation of chestnuts from The American Chestnut Cooperators' Plantation established in 2009. Survival was good, although several trees had succumbed to chestnut blight.

A cooperative project was initiated with Dr. Sandra Anaganostakis, Connecticut Ag. Experiment Station, to contrast field performance of seedlings produced as bare-root seedlings vs containerized seedlings. These seedlings will be out-planted at six sites in the Northeast in 2012. Details are in Anagnostakis' report.

Gary Micksy, Penn State Cooperative Extension in Mercer County (submitted report)

Identifying potential sites/growers for outplantings. Participants at March 5 & 26 "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. One-thousand open pollinated seed were distributed to 60 individuals. Follow-up surveys utilizing the Chestnut Chatter listserv were sent out on 09.21.11. 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 2011. Two new test/demonstration orchards including open pollinated and F_1 seedlings were established near Knox, and Sharpsville PA.

Collection of Local Genetic Materials.

- Collection and processing of local Chinese pollen
- Controlled pollination to produce F_1 seed on three American chestnut trees in Haun Orchard, Sandy Lake, PA on 07.08.11
- Balloon pollination of Beagle tree, Mercer, PA 07.12.2011 (Chinese pollen)

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 106 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, that was developed with assistance from Dr. Lynne Rieske-Kinney, University of Kentucky and NE-1033 participant. This fact sheet was utilized again in 2011 via Chestnut Chatter to enlist volunteers in monitoring the spread and severity of this pest in western PA and eastern OH. As a result, new infestations were reported in Allegheny, Armstrong, Butler and Clarion Counties in PA, and Ashtabula County OH.

Penn State Cooperative Extension newsletter "The Woodlander" informed 1184 subscribers throughout western PA and eastern OH of chestnut-related educational opportunities.

Chestnut pollination efforts were featured as the lead story in the Greenville Record Argus newspaper 07.12.11

Deborah Golino, UC Davis (submitted report)

Foundation Plant Services. The trees have been in the field for 4 seasons. Despite the alkaline conditions, they have put on 2-3 feet of new growth on numerous branches on each tree. No requests for budwood this year but one request for nuts. Varieties in the collection: Marron Comballe, Marrone di Chusa Pesio, Marrone di Marradi, De Coppi, Eaton, Campbell NC-8, Luvall's Monster, Quing.

Business Meeting

There was no official business meeting. Most of the discussion centered around the Fifth International Chestnut Congress to be held September 4-8, 2012 at the National Conservation Training Center, Shepherdstown, West Virginia 25443-4024 (http://training.fws.gov). The meeting was initially scheduled to end Friday, September 7, but the committee members felt strongly that the meeting should conclude on Saturday, September 8 at noon. The deadline for lodging and meals is July 1, 2012 with meeting registration/abstracts due June 15, 2012.

The cost of lodging at NCTC for four nights is $512/person. There was some interest by committee members to have a 50% reduced registration for students. There was consensus not to have concurrent sessions. Posters could be viewed in the hallways. There was discussion as to paying some expenses of invited speakers. Bill MacDonald agreed to contact ISHS to see about the cost of the publication. If ISHS will cover most of the publication costs, consideration of paying some speaker expenses is possible.

Dennis Fulbright commented that there are a number of agencies that might pay NE-1033 to showcase their wares—TACF®, Northern Nutgrowers Association, several chestnut cooperatives. Andy Jarosz suggested starting the meeting on Tuesday afternoon and have the keynote speaker on Tuesday evening. Depending upon the number of papers, an evening session may be necessary. Wednesday was suggested as a possibility for an evening session.

There may be a guided tour of Washington, DC Saturday morning. By the June 15 registration deadline, we will be able to gauge how much interest there is for a Saturday tour. It was suggested to use an outside contractor for the tour. It is possible that the meeting talks may end Friday night. ISHS may have their own planning session which may direct Saturday morning.

Sandra Anagnostakis volunteered to chair the history session, if necessary. Sessions will be:

- Breeding/Genetics/Genomics
- Cryphonectria/Hypovirulence
- Pests and Pathogens
- Propagation and Orchard Management
- Ecology/Reforestation
- Food Science
- Marketing

There were suggestions for invited speakers:

- Roberto Botta—Turin, Italy for genomics
- Umit Serdar—Sanson, Turkey—propagation and orchard management
- Bao Chen (Naning, China) or Nobuhiro Suzuki (Okayama, Japan)—hypovirulence
- Ling Qin (China)—orchard production or genetics

There was discussion about pairing an international speaker with a member of the NE-1033 committee for each session.

Fred Hebard suggested a preliminary announcement with a few details would be useful to the chestnut community.

There was discussion about a keynote speaker. Several suggestions were made:

- Hill Craddock on the history of chestnut in the United States
- Someone from the Smithsonian to talk about global warming

A speaker for Friday night was discussed. Retired Republican senator Christopher (Kit) Bond of Missouri was suggested as a possibility. Mark Coggeshall volunteered to make the contact.

Dennis Fulbright asked if the kitchen at NCTC will cook chestnuts if they are supplied.

Committee volunteers include: Don Nuss; Paul Sisco; Brad Hillman; Sandra Anagnostakis; Dennis Fulbright; Fred Hebard; Jeanne Romero-Severson; and, Hill Craddock.

Other business: Paul Sisco asked that those individuals who have received grants from TACF® should submit articles for publication in TACF®'s Journal.

Lynne Rieske-Kinney volunteered to host the 2013 meeting in Kentucky.

The meeting adjourned at 5:30 pm on Saturday, October 29, 2011.

Respectfully submitted,
Mark Double
West Virginia University
December 2011

2010-2011 Publications

Baidyaroy, D., G. Hausner, D.W. Fulbright and H. Bertrand. 2011. Mitochondrial plasmid-like elements in some hypovirulent strains of *Cryphonectria parasitica*. Fungal Genetics and Biology 48:764-774.

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Choi, G.H., A.L. Dawe, A. Churbanov, M.L. Smith, M.G. Milgroom, D.L. Nuss. 2011. Molecular characterization of vegetative incompatibility genes that restrict hypovirus transmission in the chestnut blight fungus *Cryphonectria parasitica*. Genetics (in press).

Clark**,** S.L., S.E. Schlarbaum, A.M. Saxton and F.V. Hebard. 2011. Making history: Field testing of blight-resistant American chestnut (*Castanea dentata*) in the southern region. In: (Fei, S., Lhotka, J.M., Stringer, J.W., Gottschalk, K.W. Miller, G.W. eds.) Proceedings, 17th Central Hardwood Forest Conference; 2010, April 5-7, Lexington, KY. Gen. Tech. Rep. NRS-P-78. Newtown Square, PA: U.S. Department of Agriculture, Forest Service, Northern Research Station: 656-657.

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Donis-Gonzalez, I., E.T. Ryser, D. Guyer and D.W. Fulbright. 2010. Shell mold and kernel decay of fresh chestnuts in Michigan. First European Chestnut Conference. Acta Horticulturae 866:353-362.

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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 Gβγ- 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.

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,106:17927-17932, 2009). Four 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.

2011

- **Identify, clone and disrupt *C. parasitica vic* genes.**

Five of the *C. parasitica vic* loci were previously linked to molecular markers on a genetic linkage map (KUBISIAK and MILGROOM, 2006). We hypothesized that, if the *C. parasitica vic* system resembles the non-self recognition systems that operate in *N. crassa* and *P. anserina,* then the *C. parasitica vic* loci should be identifiable as regions of hypervaribility located near the linked markers. We confirmed this prediction by identifying seven candidate polymorphic genes associated with four *vic* loci through comparative analysis of the genome sequences of two *C. parasitica* strains, EP155 (reference genome sequence) and EP146, that were genetically determined to have allelic differences at *vic2*, *vic4*, *vic6* and *vic7*. A role in restriction of virus transmission was demonstrated by disruption of the polymorphic candidate genes associated with the *vic* loci previously implicated by genetic analysis as restricting virus transmission, *vic2*, *vic6* and *vic7* (CORTESI *et al*., 2001). Non-allelic interactions between two tightly linked genes at the *vic6* locus were shown to trigger incompatibility and influence the frequency and symmetry of virus transmission. RNA silencing was recently shown to serve as an effective antiviral defense mechanism in *C. parasitica* (SEGERS *et al*., 2007; ZHANG *et al*., 2008; SUN *et al*., 2009b). The results of this study also strengthen an emerging view of the complementary nature of RNA silencing and the *vic* system in fungal antiviral defense at the cellular and population levels, respectively.

Completion of milestones not proposed.

2010

- The *C. parasitica* gene *oah*, encoding the enzyme Oxaloacetate acetylhydrolase (OAH), a member of the PEP mutase (PEPM)/isocitrate lyase (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 suggests that the enzyme plays a key role in virulence.
- 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.
- 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.
- Demonstrated that RNA silencing serves as an antiviral defense mechanism in *C. parasitica* (first example for any fungus) against hypoviruses and mycoreoviruses.
- 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.
- 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.