NE-1015 Technical Committee Meeting
Biological Improvement, Habitat Restoration, and
Horticultural Development of Chestnut by Management of Populations, Pathogens and Pests

Hungry Mother State Park, Marion, VA
September 7-8, 2007

Attendance:

California:	Debora Jacob-Wilk (UC Davis)
Connecticut:	Sandra Anagnostakis (Connecticut Agricultural Experiment Station)
Georgia:	Scott Merkle (University of Georgia)
Kentucky:	Lou Shain, retired (University of Kentucky)
Maryland:	Donald Nuss (University of Maryland Biotechnology Institute); Robert Strasser (Hood College)
Michigan:	Dennis Fulbright, Irwin Ronaldo (Michigan State University)
Mississippi:	Tom Kubisiak (USDA-FS)
New Jersey:	Bradley Hillman, Sara Baxer, Jo Anne Crouch (Rutgers University)
New Mexico:	Angus Dawe—chair, Amanda Kemp, Rong Mu (New Mexico State University)
New York:	William Powell, Nick Kaczmar, Lilibeth Northern, Kathleen Baier, Amelia Bo Zhang (SUNY-ESF), Michael Milgroom (Cornell University), Steven Jakobi (Alfred State College)
North Carolina:	Paul Sisco (TACF, Asheville); Ron Sederoff (NC State)
Ontario:	Adam Dale (University of Guelph)
Pennyslvania:	Ali Barakat, Kelly Deitrick, Scott Diloreto, (Penn State University), Sara Fitzsimmons (PSU, Regional Science Coordinator, TACF)
South Carolina:	Laura Georgi, (Clemson University)
Tennessee:	Hill Craddock, Meagan Binkely (UT Chattanooga); Scott Schlarbaum (University of Tennessee)
Virginia:	Fred Hebard, Bob Paris, William White (TACF, Meadowview)
Washington:	Nicholas Wheeler (Molecular Tree Breeding Services, Centralia)
Washington DC:	Bob Nowierski (USDA-CSREES)
West Virginia:	William MacDonald, Mark Double, Shawn Kenaley (West Virginia University)

The meeting was called to order by Chairman Dawe at 8:00 am on September 7, 2007 at Hemlock Haven Conference Center at Hungry Mother State Park in Marion, VA. Dawe thanked Fred Hebard, Bob Paris and William White for their assistance with local arrangements. Dawe introduced Bob Nowierski from USDA-CSREES and thanked his students, Amanda Kemp and Rong Mu for their help with registration.

Bob Nowierski-USDA-CSREES

Nowierski indicated that the Cooperative State Research Extension and Education System (CSREES) is a federal partner for the land grant university system. They

administer nearly $0.25 B in competitive grants. They also administer congressional earmarks and formula funds such as Hatch dollars and McIntyre-Stennis funds. CSREES also reviews departments and develops white papers that set the direction for national research funding. Nowierski apologized for this being his first meeting, but he has 11 multi-state committees for which he is the federal liaison. He has to focus on committees that are up for renewal and NE-1015 is in that category. Since this project is up for renewal, he cannot emphasize enough the importance of coming up with strong impact statements which are used to justify the maintenance of formula funds.

Nowierski commented that he looks forward to meeting members of the group and listening to our research reports so that he can better represent us in Washington DC. He also stated that he would be happy to discuss funding opportunities.

<u>OBJECTIVE 1</u>. To improve chestnut trees for reestablishment in forest ecosystems, and chestnut cultivars for nut production by selection, breeding, and marketing, and determine the cultural criteria of all chestnuts for successful production in nurseries, orchards, and/or natural settings.

Sandra Anagnostakis—Connecticut Agricultural Experiment Station

Hybrids backcrossed to American chestnut. Japanese BC3 hybrid trees were seeded in 1997 both in clearcuts and in a forest setting. The trees are now nine years old and 25' tall in the clearcut. She noticed ozone damage on the leaves of these trees. The damage is evident on the tops of the leaves only. She indicated that she has been planting trees in clearcut for many years. The native sprouts that come up in the clearcuts are treated with hypovirulent strains that have been matched to the native *C. parasitica* population. Hypovirulence is keeping those sprouts alive. She will be collecting nuts from native sprouts and doing peroxidase testing to look for evidence of hybridization. Anagnostakis showed data from four families of backcross trees that are growing both in open field and clearcut settings. The mean dbh shows that while the clearcut trees were larger initially, the field trees are catching up, in terms of size.

Backcross with Ozark chinquapin. An Oklahoma Forest Service employee told Anaganostakis years ago that chestnut blight was rapidly killing off the Ozark chinquapin in the Ozark plateau. Anagnostakis is conducting this experiment with Scott Schlarbaum at the University of Tennessee and Stacy Clark at Bent Creek, NC. A map from George Johnson showed that the majority of Ozark chinquapins are in the Ozark plateau. Some Ozark chinquapins can be 2' dbh and 60' tall; this is quite different from the small, shrubby appearance of Allegheny chinquapin. Anagnostakis showed pictures of the Ozark chinquapin planting in Oklahoma and one thing she noticed is that the burrs develop and the branches continue to elongate 2-3' passed burr formation. This is unlike Chinese, Japanese and American chestnut where the burrs develop at the ends of branches. She is using *C. henryi* (Chinese chinquapin) in this backcross program, a species fairly resistant to chestnut blight. Scott Schlarbaum and Stacy Clark will be planting out some of the hybrids that include:

C. henryi X *C. ozarkensis*
C. ozarkensis X *C. henryi*
C. crenata X *C. henryi*

In this study, Schlarbaum and Clark are looking for resistance to chestnut blight and form of Ozark chinquapin.

Gall wasp study, in conjunction with Stacy Clark and Henry McNab (USDA-FS). In 1995, 93 trees were planted representing: *C. dentata* X (*ozarkensis*mollissima*) and *C. dentata* X (*pumila*crenata*). These backgrounds were used because Jerry Payne was convinced that the native chinquapins had resistance to gall wasp. Anagnostakis and Payne both believe that American chestnut is totally susceptible to gall wasp. These crosses will allow them to determine if gall wasp resistance, if it exists, is cytoplasmically inherited. Since the female parent is susceptible—if there are any resistant offspring then resistance is not cytoplasmic. Henry McNab put in the planting and tended it and Stacy Clark took the data. The trees have been in the site for 11 years. Some of the trees have chestnut blight and others do not. Clark scored each tree for the presence or absence of chestnut blight. Control trees were planted and they all died. There was better survival on the Allegheny chinquapin with the Japanese background than in the Ozark chinquapin with the Chinese background. There were a number of trees that had few galls while other trees were heavily galled. Anagnostakis was interested if there were any trees that had little or no chestnut blight along with few, if any, galls. Since there were trees that had few galls, the inheritance is not cytoplasmic. This is only 11 years of data and that is not sufficient. She hopes that Henry McNab will continue to tend to the trees so that they might be able to examine the next generation of trees since the trees are beginning to produce seedlings in the area.

Oriental chestnuts in VA. Anagnostakis stated that NE-1015 members might be curious if the chestnuts growing in Marion, VA are actually native chestnuts. She reviewed the USDA records of other chestnut species that were shipped to VA. From 1935-1953, nearly 350 trees, mostly Chinese chestnut, were sent to Marion, VA. From 1935-1938, 130 chestnuts, both Chinese and Japanese, were shipped to Abingdon, VA. Hebard indicated that there are Chinese and Japanese chestnut scattered throughout the county. Anagnostakis volunteered to supply members with USDA records for particular areas of interest. She sent USDA records to Hill Craddock with regard to chestnuts that were planted near his planting in Georgia.

Scott Merkle—University of Georgia

American chestnut somatic seedling production for restoration. Merkle indicated that he almost quit chestnut research about six years ago because he had run out of funding and they had experienced very little success—only five somatic embryos after ten years of research. He kept his cultures viable and additional funding was the impetus for his renewed work. He presented work that was the result of his technician and students, Gisele Andrade, Sara Johnson and Taryn Kormanik. Merkle discussed the rationale for development of an *in vitro* propagation system for American chestnut as:

- Mass clonal propagation of promising genotypes generated by breeding programs (TACF, ACCF)
- Provides a means of regenerating trees engineered with anti-fungal candidate genes
- Helps conserve American chestnut genetic diversity via cryostorage

Current state of somatic embryogenesis and transformation research at UGA:

- Cultures have been initiated from full-sib material

- Improvements in somatic embryo production have been made
- Improvements in somatic seedling regeneration have been made
- Somatic seedling performance in the nursery is being examined

Culture Initiation from full-sib material—The problem was that all cultures initiated up to 2006 were derived from open pollinated material, and thus of unknown male parentage. Their approach was to culture full-sib immature seeds from controlled pollinations (CP)

  - Crosses between pure American chestnut parents; this collection was coordinated by Sara Fitzsimmons from TACF members who conducted controlled crosses of known American parents.
  - Crosses using advanced generations from TACF backcross material

American chestnut induction-maintenance medium recipe:

- Lloyd and McCown's Woody Plant Medium major salts (N, P, K, Ca, Mg)
- Lloyd and McCown's Wood Plant Medium minor salts (Mn, Zn, Cu, B, Mo)
- Murashige and Skoog's Iron (Fe)
- Schenk and Hildebrandt's vitamins (Thiamine, nicotinic acid, pyridoxine)
- 30 g/l sucrose
- 1 g/l casein hydrolysate or 0.5 g/l L-glutamine
- 2 or 4 mg/l 2,4-D (auxin)
- Gelled with Phytagel gellan gum

Undergraduate Sara Johnson conducted an experiment to determine if higher concentrations of 2,4-D or a different plant growth regulator (PGR) will improve initiation frequencies. She set up 11 Families – some controlled pollinations with 4 PGR treatments: (1) 2 mg/L of 2,4-D; (2) 4 mg/L of 2,4-D; (3) 2 mg/L of picloram, another auxin; and, (4) 4 mg/L of picloram. Data indicated that there was variation by family and that there was no reason to vary from their standard of 2 mg/L of 2,4-D.

If possible, they start with embryos from seeds that are very mature. Embryonic cultures begin very small (1.5 mm-diameter). There are 12-18 embryos/seed. If they are lucky, 3% will begin to proliferate. Over time, they can get massive amounts of somatic embryos. If they continue to grow them on semi-solid media, they are all stuck together. When an attempt is made to pop them off to germinate them, the embryos are damaged. Much higher quality embryos can be obtained in shake cultures. Cultures must be fed every two weeks or they turn dark. Only 5-10% of the cultures they have begun over the years can make a nice suspension culture. They use a protocol, originally developed for yellow poplar—shake cultures are size fractionated through 140 μm and 38 μm screens. They can end up with about 2,000 globular-stage embryos in a culture flask. If the embryos are plated out from the fractionated material, it is easy to pick high quality embryos that are not damaged.

In order to get larger embryos prior to germination, they tried various means:

- activated charcoal
- carbohydrate (sucrose, maltose, fructose)
- amino acids (asparagine, glutamine)
- abscisic acid
- polyethylene glycol
- cold stratification
- desiccation

The only treatments that were useful were activated charcoal, changing to glutamine and cold stratification. Their embryos required 12 weeks in cold storage prior to germination. Activated charcoal makes a difference although it is a subtle difference—it keeps the roots white and encourages branching. Lateral roots are very important for survival once they are placed in pots. With the work of Gisele Andrade, they now have a system whereby they can get plants from embryos. Sara Johnson began testing longer cold treatments to try an increase germination and conversion. (Germination is defined as radical elongation into a root while conversion is an embryo that goes on to form a complete plant that can be transplanted to potting mix and survive). Johnson used three embryonic lines and five cold treatments, some up to 18 weeks. She found that there was an interaction between genotype and treatment. Some genotypes liked 18 weeks of cold treatment and other lines did not. Some lines geminate only with 15 weeks of cold, not 12 and not 18. These data indicate that for every clone, conditions will have to be optimized. Light quality also was examined. They used three lines and three light treatments: (1) cool white fluorescent light (100µmol m-2 s-1); (2) red (600 nm) from LEDs; and (3) red + far-red (735 nm) from LEDs. Overall, germination and conversion can be increased with red light treatment. While some clones like red light and other do not, they have changed their standard protocol to red light, from cool white.

Taryn Kormanik began looking at different potting mix, including nursery soil, and fertilizers. Their standards had been Fafards and Hoagland's solution, but Kormanik began looking at Miracle Gro. Better looking plants were obtained from Fafard and Miracle Gro than with Farard and Hoaglands. Many of the seedlings from the fertilizer study were outplanted at the Georgia Forestry Commission's Flint Nursery. Many of the seedlings were browsed by deer, but the few that were not browsed were in excess of 5' tall after only one growing season. While the seedlings looked good, many of them had root problems. For whatever reason, the tap root was lost and only lateral roots remained. According to Kormanik, seedlings with only lateral roots will survive in the nursery but they will not survive when outplanted in the field. Kormanik's problem is to find out what is going on with the root system of these seedlings—do they start off with a tap root? Do they keep the tap root? What makes them lose the tap root? Kormanik has been looking at micrographs this summer and some somatic embryos have nice root meristem. There are a lot of somatic embryos that have full development of their cotyledons, but she cannot find a shoot or root meristem. When Kormanik finishes documenting what is going on, they will try and prove what is going on with the meristems.

Transformation experiments were conducted by Gisele Andrade who examined the following variables:

Variables:
-genotype (4 plant-forming embyogenic lines
-days between fractionation and co-culitvation (2-4 days)
fractionated PEMs
-*Agrobacterium* OD
-co-cultivation length (2 or 3 days)

Other Factors:
-*Agrobacterium* strain AGL1
-plasmid pCAMBIA 2301
-target material was 50 mg size-

-*uidA* (GUS) reporter gene

Selection:

-Antibiotic type (kanamycin, genticin, hygromycin
-Antibiotic concentration

They would like to eliminate the reporter gene and not use GUS. Merkle showed slides of transgenic somatic embryos. They will have to screen through lines to see which transgenic lines have a decent conversion rate. Andrade has a pretty solid transformation system. Andrade's PCR work shows that in some cases they have the nptII gene, but the GUS gene is gone. While they are both on the same plasmid, they do sometimes lose one of the genes.

About nine months ago, they shifted from working with reporter genes to constructs with anti-fungal potential. Merkle is working with new vectors (pWVR31, pTACF6, ESF39A) from ArborGen in three American chestnut genotypes. They have their first pTACF colonies (no plants yet), driven by the same promoters as GUS. The control embryos do not have the antifungal genes. They are hoping to crank-in these antifungal constructs.

Adam Dale-University of Guelph

Colin McKeen stepped down as chair of the Canadian Chestnut Council (CCC) in 2005. Colin is now 86 years old and he has been chair of the council since its inception in 1988. Dr. McKeen's initiatives led to the establishment of a breeding program in 2002 and he received two grants from the Trillium Foundation to support his breeding initiative. McKeen continues to serve on the board. The new chair is Terry Anderson, a soybean pathologist.

In Canada, American chestnut is native to only SW Ontario. It was assumed that there were only a few hundred American chestnuts in Ontario, but a survey of public lands by Greg Boland, John Gerrath and Brian Husband documented well over 1,000 trees. There has been no surveys of private lands so they believe they have a healthy population. One big concern for a breeding program that involves chestnut--Are chestnuts that are thought to be pure American chestnuts actually pure? John Gerrath, a MS student, looked at various hybrids, both morphologically and through RAPDs. He identified 3-4 RAPDs to distinguish species. Ones that were thought to be hybrids via morphology, may not actually be hybrids. Thus, the population in Ontario may be true American chestnut.

An ambitious breeding program was established with the goal of have American chestnuts that are 75% Canadian origin in 20 years. Anagnostakis provided pollen for the study and they now have a population of 838 seedlings from Anagnostakis' material. Canadian environmentalists will not be happy with anything that has foreign origin, so they have been conducting Canadian X Canadian crosses just to maintain the germplasm. They now have 805 trees of Canadian hybrids in the field. The plan was to use these Canadian chestnuts in their controls for inoculations with *C. parasitica*. In a second Trillium Foundation grant, they reviewed their goals. First, they are still using Anagnostakis' material. Second, they are looking at the Canadian X Canadian crosses for any resistance that may develop. Third, they have noticed that many of the parent trees in the forest are dying so they set up a program to graft material and make sure it is in plantations.

They are very fortunate to have the cooperation from the Tim Horton Foundation. The Horton Foundation has given the CCC 30 acres for chestnut planting. There is a second planting SW of the Horton planting, complete with deer fence, for chestnut work. The first trees in the plantings were large enough to begin inoculations. They debated whether to do trunk or branch inoculations. They decided on branch inoculations and the size of the lesions varied from 30mm on resistant trees to 60mm on susceptible trees. There was a normal distribution of cankers sizes. Thus, it looks like there is some resistance in the pure American that they might be able to exploit.

The CCC is trying to maintain trees in the forest so they have the germplasm around for use. They have been having an awful time with grafts. Either they do not take or they take and after 2 years in the field, the grafts crack and the trees die. His technician is convinced that they can get cuttings to root directly. He has been working on this problem and he feels that, with the proper material, he can get 20% of the cuttings to root directly. If they can get a figure of 20% to root directly, it will save them a lot of agony with grafts. They are continuing to work out the details of this problem; the trick is to get shoot production from the terminal bud in the first year.

Dennis Fulbright—Michigan State University

There are a number of chestnut sites in Michigan that are recovering. They have been working in a number of these sites inoculating with the Michigan hypovirus, CHV3. In the 1980s, they began spreading the hypovirus in one particular site. Last year he reported that after 20 years, this site is now recovering. This year, isolations were made from cankers and the resulting isolates were hypovirulent. The isolates appear to reduce the aggressiveness of virulent strains and alter their morphology. They have yet to determine if these hypovirulent isolates are CHV3-GH2.

In the 1990s, chestnut growers in Michigan were told to plant Chinese chestnuts to start orchards. The questions that followed were a true test of the land-grant system. The questions came from the growers to the extension service to the university. The growers asked their extension agents, how to grow chestnuts. The extension agents then posed the same question to the university and Fulbright wanted to know what to tell the growers? The last NE-1015 grant proposal incorporated a good objective for growing chestnuts.

He showed pictures of trees recovering at the Crystal Lake site—lots of roughened bark. Cankers cannot be readily defined except for young trees. Hypovirulent strains, mostly GH2, were released and they can be recovered. Under objective one of the proposal, they set up a chestnut growers' chestnut cooperative and assisted with developing a market. They are still trying to determine what a chestnut orchard in Michigan should look like. The growers were told to plant seedling chestnuts and that advice came from lay groups (i.e. MI nutgrowers' association). The MSU group advised growers to begin planting chestnut cultivars. If diversity is a goal, the diversity should come with the cultivars. There are 37 growers in the cooperative. Fulbright showed pictures of a MI chestnut grower that is harvesting the goal of 2,000 pounds of chestnuts/acre—primarily the cultivar 'Colossal'. Irrigation and weed control are utilized and it is looking pretty good for chestnut production in MI.

They are looking for new cultivars that will thrive in the MI climate. A new cultivar, 'Labor Day' will be released. It drops its nuts in early September; this is a good

cultivar for northern MI. Chestnuts in northern MI get hit by sleet and cold rain in late fall, so an early nut is beneficial. Other chestnuts are being studied, most notably a dwarf chestnut from Korea. Everything on the dwarf is smaller, but the nuts are normal size. It is male sterile, so it does not add any pollen to an orchard. One advantage is that it is easy to manipulate, especially for spraying with a backpack sprayer.

Michigan chestnut growers received a $100,000 check from the Michigan Dept. of Agriculture. They received a very large value-added grant from a state that does not have a lot of money to give away. In addition to the MI Dept. Ag. grant, the MI chestnut growers, in their six years of existence, have been subsidized by the following: (1) a $100,00 chestnut peeler; (2) two value-added grants; (3) new specialized slicing machine; (4) Kerian sizer; (5) Fulbright's expertise; and, (6) collection, storage and processing at MSU facilities.

Irwin Ronaldo Donis González-Michigan State University

Gonzalez, a graduate student from Guatemala, reported on his work with post-harvest spoilage on chestnuts in Michigan. His objectives are to: (1) determine the post-harvest obligate pathogens that are associated with fresh chestnuts; (2) determine the non-obligate parasites associated with spoiled fresh and mechanically peeled chestnuts; and (3) study the ability of different antimicrobial agents to reduce pathogens and spoilage organisms during storage of fresh and mechanically peeled chestnuts. Spoilage was defined as the deprivation of a product of good or effective qualities making it no longer acceptable. Spoilage is the result of insect damage, moisture loss, discoloration, physiological disorders or microbial activity. Post-harvest diseases that cause spoilage are world-wide. Losses are about 25% and these losses can be quantitative or qualitative. Losses may be greater than the economic gains that are achieved by improvements in production. Studies on post-harvest diseases are primarily directed to prevent economic loss and to eliminate the negative effects of mycotoxins. Using ELISA, they are able to detect the following mycotoxins on chestnuts in MI: zearalenone, DON and ochratoxin. Chestnuts, in general have:

- Relatively high moisture content
- Low fat (oil) content
- Thin-permeable shell
- Dry out rapidly

After harvesting, chestnuts are more similar to a fruit than a nut. They are susceptible to damage, microorganism and insect attack and desiccation.

Fungal rotting is the most serious post-harvest problems in chestnuts around the world. The most important are:
Penicillium spp., *Botryris cinerea*, *Phomopsis castanea, Fusarium* spp., *Ciboria batschiana* (=*Sclerotinia pseudotuberosa*). Various species of yeast are found to colonize chestnuts.

Penicillium can cause spoilage on the hilum which can rot the kernel. *Fusarium* creates a white mantel in groups of chestnuts; it does not enter the kernel but those nuts are rejected by customers. Kernel and wet rot can be caused by bacteria and fungi. Some of the yeast include species of *Cryptococcus* and *Candida*. He quantified the number of microorganism on fresh chestnuts and found that for fungi and bacteria, the peak of contamination occurred at 90 days when stored at 4C. When chestnuts fall to the ground,

there is a 3-4 fold chance of getting contaminated than if they were harvested directly from the tree. There is more mold on the hilum than on the shell or kernel. More bacteria and yeast are found on the hilum and kernel as well. The amount of microorganisms (bacteria, mold and yeast) increases significantly during 90 days of storage (4°C); then stabilize; and sometimes decreases or increases after 120 days.

Meagan Binkley— University of Tennessee-Chattanooga

Binkely is working with Hill Craddock, Fenny Dane and Joey Shaw to look at natural chestnut-chinquapin hybrids. They are confounding taxonomy and she is using a DNA sequence-based inquiry into this putative hybrid populations. Binkely showed range maps of American chestnut (*Castanea dentata*), Allegheny chinquapin (*C. pumila*) and Ozark chinquapin (*C. ozarkensis*). There is some overlap of *C. dentata* and *C. pumila* but *C. ozarkensis* is unique in its distribution in the Ozark mountains. Historically, these species have been problematic. There is some historical evidence for hybridization. The problem is that putative hybridization in the southeastern US is confounding the TACF's breeding efforts. Some trees have leaves like American chestnut but they have the fruit of Allegheny chinquapin. There are four hypotheses involving these strange trees:

1) Variable chinquapin morphology
2) Chestnut x chinquapin
3) Asiatic hybrids
4) Lost population of Ozark chinquapin

The goals of her work are to:

1) Identify a chloroplast marker that can be used to separate *C. dentata, C. pumila*, and *C. ozarkensis*
2) Use DNA marker (PCR-based or DNA sequence-based approach) to differentiate between these species at the molecular level
3) Test a confounding "Pocket" population in northern Georgia to determine the reason for its uniqueness

To date, her fieldwork is complete. She has collected 226 accessions of American chestnut and chinquapin from her study sites in Walker and Floyd Counties, GA and Monroe and Polk Counties, NC.

She chose to work with a chloroplast marker because they evolve much more quickly than mitochondria. She is using a PCR-based screening method to look at the *trn*T-L that Fenny Dane and Tom Kubisiak described in 2006. There is a 75 bp indel unique to *C. dentata;* this as a region that separates *C. denata* from *C. pumila* and *C. ozarkensis.* She conducted a small study using 10 samples from NC and 10 from TN. All of the samples lacked the indel; they are more like chinquapin than American chestnut. She looked at a sympatric population from Sherwood Estates, NC where chestnut and chinquapin maintain their distinctness. Her data were confusing as she found chestnuts and chinquapins with and without the indel. She will screen more accessions and compare morphological characters with her chloroplast marker.

Hill Craddock—University of Tennessee-Chattanooga

Craddock commented that chestnut breeding work is exciting and beautiful work, partly because of the places they get to go to. He showed a slide taken from a bucket

truck of the Cherokee National Forest that sits on the border between NC and TN. There is a population of naturally occurring chestnuts in the forest, many of which bloom. They have been released due to an attack of pines by southern pine beetle. Many of the trees at this site have swollen cankers, typical of those caused by hypovirulence. Some of the trees are still blight free. He showed pictures of the variable morphology of chinquapins from a "pocket" population in northern Georgia. He stated that it is not far-fetched to believe that there may be Ozark chinquapin in northern Georgia—just because it is not on a distribution map does not mean it is not there. Craddock stated his home county, Hamilton, is not listed on the range map for American chestnut, but there is plenty of American chestnut in Hamilton County.

Craddock shared some of the highlights from the breeding season, from the viewpoint of the TN chapter of TACF. The TN chapter participated in the father tree program. Rather than the typical scenario whereby the TN chapter gets pollen from the TACF group in Meadowview, this year, Craddock collected catkins and pollen and sent it to Meadowview. There are a couple of reasons for this. First, the southern chestnut trees tend to bloom before the pollen from Meadowview is ready. They often use stored pollen and sometimes it works and sometimes is doesn't work. Second, it is a logistical problem to get pollen out in the wilderness; it is actually more convenient to get pollen from trees in the wilderness and take it to Meadowview. It is easy to work at Meadowview as the trees are all together and working off ladders at the farm is not a problem. Many of the southern states contributed to the father tree program—AL, GA, KY, NC, SC and TN.

Craddock has a family of B2F2s that were intercrossed at Meadowview. Hebard sent Craddock the seeds in 1996 and they were planted in Chattanooga. The trees have been screened and selected; Craddock is using those to make an F3 controlled pollination onto clones of a Meadowview B2F2. Craddock is using vegetatively propagated clones; he has a whole orchard of TACF hybrid material. There are interesting cultivar differences. He showed a picture of two clones, one which bloomed this year (clone SA419) while clone SA408 did not bloom (the trees were the same age).

Craddock indicated that he wanted to talk about the state of the commercial chestnut industry in TN but there was a catastrophic freeze in TN, April 7-9 following two weeks of warm weather. All of the Chinese chestnut cultivars were destroyed. In his cultivar trial, all 20 trees of the cultivar 'Amy' were killed to below the graft union.

Craddock showed pictures of the Bendabout Farm B2F2s that were planted in 1996 and screened in 2003. The trees were inoculated with Ep155 (virus-free); in addition, they were inoculated with two hypovirulent strains, CHV1-Euro7 and CHV1-Ep713. Ep155 was used for the resistance rating. Cankers were measured in mm. The most resistant trees, I-8 and I-11 are nearly 40' tall.

Bob Paris—The American Chestnut Foundation, Meadowview

TACF resistance program. Paris reported on some of the newer projects that they are involved with at Meadowview since he began a few years ago. He hopes to begin making progress on some of these projects shortly. Paris wants to look at resistance in the breeding program at Meadowview. The main sources of resistance in the Meadowview backcross breeding program are 'Clapper', 'Graves' and 'Nanking'. While there are other sources of resistance in the pipeline, the three aforementioned are

the main sources. Most of the advanced material currently is from 'Clapper' and 'Graves'.

Paris posed the question, 'How do we go about finding additional sources of resistance?' Other Chinese chestnut trees that have suitable resistance can be used; there are numerous Chinese chestnuts at the Meadowview farm. Other sources might be some of the original introductory material at USDA facilities. China is also another source of trees with resistance. Eighteen Chinese chestnut tress with good resistance were identified at the Meadowview farm. Those trees were crossed with 'Mahogany', 'Meiling' and 'Nanking'; these three closely represent the three sources of resistance in the program. Progeny will be screened for resistance. They are also looking at Japanese chestnut and large, surviving American chestnuts. They hope to focus not solely on Chinese but use any tree that offers some sort of resistance. He hopes to begin compiling some of these trees to add to the breeding program.

Both the progeny and parents need to be examined, in terms of resistance. In February, they took open pollinated Chinese chestnuts out of the cooler and germinated them in the greenhouse. This was Paris' first up-close look at germinating chestnuts. Radicals grew down and after about 10 days the stems emerged. At 4" in height, Paris began to do chip bud grafting. After a good while (20 weeks), they could tell if the bud was going to make it. Many of the chip bud grafts died; those that survived were destroyed by squirrels. A few did make it to the field. About the same time they took the nuts out of the cooler, they began planting the progeny in the field. About May, the parents that had been grafted in the greenhouse were planted among the progeny in the field. After a few months, they began noticing that there were quite a number of bare spots in the planting scheme—no trees were evident. That prompted them to look at their planting methods and they questioned the staff. They found out that one girl planted her nuts upside down and those trees did not survive. Generally, their planting crew does a good job.

What they hope to get out of this study is an evaluation of what their germplasm has in terms of resistance. They also hope to ascertain what they can expect out the breeding program.

Cropping history experiment. They are evaluating the performance of chestnut following corn, tobacco and grass. How does cropping history affect chestnut growth? When the time comes for TACF to begin distributing nuts to the public for planting, they would like to be able to make some recommendations. They assume that not all of the nuts will make their way to the forest, but some of the nuts may be planted lands that were formerly cropped. Three major crops in southwest VA are corn, tobacco and grass. The established plots containing these three crops; after three years, they will plant chestnuts to see how chestnut performs following these crops.

William White—The American Chestnut Foundation, Meadowview

Pollen germination. One of his main jobs at the Meadowview farm is to collect pollen and send it off to the state chapters. In past years, they have found no correlation between percent pollen germination and nut yield. White wanted to optimize the pollen germination assay so that he could: (1) provide viable pollen to state chapters; and, (2) detect any genetic abnormalities. Last year, he used a moisture chamber method of germination but that method produced a lot of aborted pollen. He researched several

techniques to look at pollen germination. A hanging drop slide using an optical depression microscope slide gave better results than the moisture chamber method. He also experimented with various concentrations of sucrose. The method he settled on was: use of hanging drop, room temperature distilled water, 4 mg pollen per 1.5 ml water, a 0.5% sucrose concentration. All of this culminated in a 38-39% germination rate in 2007. Specifics of the method can be found at the website: www.acffarms.org/papers/Pollen%20germination%20assay.pdf.

Scott Schlarbaum—University of Tennessee

Schlarbaum is responsible for the tree improvement program at the University of Tennessee Agricultural Experiment Station; chestnut is only a small part of this project that centers mostly on oaks. He teaches a class on the role of forestry and forests in American society—essentially a history class, and he often reviews old books and journals. He came across a writing from George Washington to Thomas Jefferson: *"This letter, handed to the care of Colo. Deakens, will be accompanied by a small bag of Spanish Chestnutts [sic], half of which you will please to accept, and the other contrive to Mr. Lee; they were sent to the Alexandria races in October to be given to him, but the delivery was neglected. It might be well perhaps to put them in sand to prevent an over drying, to the injury of vegitation [sic]."* It is obvious that in addition to being a statesman, Washington also knew how to handle chestnuts.

Chestnut seedlings at Georgia state nursery. Schlarbaum showed pictures of 1-year old seedlings that were planted at the Flint River Nursery in southern Georgia. The seedlings have been planted using the protocols of Paul Kormanik a retired US-Forest Service employee. Many of the 1-year old seedlings, from Anagnostakis' chestnut families, are seven feet tall. Schlarbaum also grows Allegheny and Ozark chinquapins at this nursery. He noted that the chinquapins are a bit gnarly in their growth habit; while neither chinquapin grows as tall as chestnut after one year, many of the Ozark chinquapins are nearly five feet tall. The Ozark species looks quite different from Allegheny chinquapin.

Open pollinated progeny tests. They have 653 seedlings from 12 families planted at four sites in PA: Clear Creek, Buchannan State Forest, Sproul State Forest and Delaware State Forest. They use pure American chestnuts because they believe they will live long enough to reveal the requirements necessary for the reestablishment of American chestnut. The seeds from this experiment were provided by the PA-TACF chapter, germinated at the Flint River Nursery and outplanted in PA. This work was done in conjunction with Tom Hall and Jim Bailey of the PA Bureau of Forestry, Sunshine Brosi (a former UT graduate student) and Ann and Bob Leffel of the PA-TACF chapter. There was a lot of mortality in 2007, but some of the trees have survived and many of those are nice looking trees. The Clear Creek, Buchannan and Sproul sites were fenced to protect against deer predation and tree tubes were used at the Delaware site which was fenced later. Clear Creek gave significantly better growth than the other three sites. There were differences among the 12 families, ranging from 150-300 cm in height. Schlarbaum hopes to have the final data from these plantings in another year or two. Another set of plots was established at two sites in KY, the Daniel Boone National Forest and a site at Berea College. Seeds for this study were supplied by Hebard. At the end of the 2000 season, there was 48.15% mortality. Of the 174 living seedling, the average

height was 127 cm while the average height growth was 25 cm. The KY sites had a significant *Phytophthora cinammomi* problem. The Flint River Nursery has a *P. cinammomi* issue and that carried over to the forest plantings. The KY sites are heavy clay and there was heavy mortality, not from chestnut blight but from *P. cinammomi*. The trees that survived grew amazingly well; they competed with the yellow poplars. The trees would do better if they were planted "fresh"; often from the time that the seedlings are lifted, measured, pruned and finally planted can be as much as two months.

Mammouth Cave National Park genetic tests. The material for this full-sib study came from the Adair County, KY tree which is one of the largest surviving American chestnuts known in the U.S. Crosses in this study include: Adair X Wayne; Adair X Clinton; Adain X Jackson; and, Adair X Wester. All the pollinations and collections were conducted by the KY-TACF chapter. The six sites for outplanting were identified by Joe Schibig of Volunteer State University in TN. These plots will be highly characterized for light, soils and other factors that might affect tree survival and growth. This is similar to precision forestry with oaks in which they try to zero in on critical factors for tree growth. Tree survival ranged from 16% to 93% among the six plots. Tree survival across family lines ranged from 38% to 77%.

UT Space Institute. The Space Institute wanted a hardwood component on some of their loblolly pine land. A 30 acre site in southern TN was used for this study. Five-foot-wide lanes were cut in the pine stand. American chestnut trees, along with white, black and northern red oak were planted; Schlabarum indicated that the chestnuts have a chance to get above the pines. This study, however, might fail due to drought.

Sunshine Brosi, Schlarbaum's former graduate student, accepted an assistant professor position at Frostburg State University in MD where she hopes to continue her chestnut and butternut work.

Michele Warmund, Michael Gold and Kenneth Hunt, University of Missouri (submitted report)

Consumer awareness. A longitudinal study was conducted during the Missouri Chestnut Roast Festivals in 2003, 2004 and 2006 assessing participants' familiarity with chestnuts, key attributes influencing purchase decisions and essential knowledge about chestnuts. Familiarity with chestnuts increased over time. Participants valued product quality, local production and nutritional value. Compared to first time visitors, repeat visitors reveal an increase in frequency of consumption, familiarity with roasting chestnuts and an increase in knowledge. Results demonstrate the positive impact of the event on participant's knowledge, familiarity and interest for chestnuts. Thus, Extension professionals can use community festivals as an effective educational tool.

Little giant and other chestnut cultivars as a source of genetic dwarfing for Chinese chestnut trees. In 2003, seed and budwood from Little Giant was collected from trees in Connecticut and shipped to the University of Missouri. In March 2004, Cropper and Little Giant seed were placed in oyster flats in a greenhouse. Seedlings were subsequently shifted to 2 gallon containers on May 15 and placed under a shade structure at HARC for the rest of the growing season. In March 2005, two scion cultivars (Eaton or Auburn Super) were grafted onto Cropper or Little Giant seedling rootstocks. Little Giant on its own seedling roots was used as a control.

In spring 2004, Little Giant budwood received from Connecticut was top-worked onto some border trees in existing plantings at HARC to produce interstem pieces of sufficient length (15 to 20 cm) to be grafted onto Cropper rootstocks the following year. In spring 2005, 20 trees of each combination were grafted:

Eaton/Cropper	Eaton/Little Giant/Cropper
Auburn Super/Cropper	Auburn Super /Little Giant/Cropper
Eaton/Little Giant	Cropper/Little Giant

Substantial grafting failure occurred due to Little Giant rootstock "bleeding." After re-grafting, few trees of Eaton or Cropper on Little Giant rootstocks were produced. Because of this grafting failure, Little Giant seedlings were produced in 2006 for evaluation as a rootstock. The other tree combinations with Little Giant used as an interstem were retained.

Following assessment of winter survival on April 1, 2006, a minimum of 10 replications of each rootstock combination was planted in a randomized complete block design at HARC in April 2006. For ten years after field planting, tree survival, trunk cross-sectional area of the scion at 5 cm above the graft union, winter injury ratings of rootstock and interstem tissue, tree height and spread, annual yield and yield efficiency data will be recorded. Key findings and outcomes**.** Burs were produced on all trees this first growing season except on those with the 'Little Giant' interstem. Nuts did not attain commercial size by harvest. Tree height and trunk circumference were similar among all trees. It is unknown if these results will continue throughout the life of the planting. While the lack of bur production in the first two years is desirable, cropping on the interstem trees would be desirable thereafter. In other tree crops on dwarfing rootstocks, such as apple and peach, fruit is generally removed in the first two years after planting to develop the vegetative structure of the tree and to prevent them from runting out. Our long-term goal in this project is to identify a suitable dwarfing rootstock that can be clonally propagated to ensure that trees produce a profitable crop of chestnuts annually.

In March 2006, chestnuts obtained from Connecticut were sown to produce rootstocks. These chestnuts include King Arthur and Hope. These seedlings were used as rootstock and grafted with Auburn Super and Eaton in April 2007. Trees will be field planted for long-term evaluation.

Thinning secondary burs of Chinese chestnut trees to enhance nut yield--Key findings and outcomes: In 2006, the following cultivars in the repository produced 2º burs on 51-75% of the main scaffold branches: Crane, Orrin, Armstrong, Douglas #1, Maraval, and Belle Epine. Moreover, this heavy production of 2º burs was apparent on these cultivars much earlier (Aug. 3) than many other cultivars that produced 2º burs later (by Sept. 3). Some trees, such as Auburn Homestead, Miller 72-76, Simpson, Carr, and Miller 72-105 did not produce any 2º burs this season. Since 2004, there has been only one growing season (2004) in which none of the chestnut trees produced 2º burs. The lack of secondary flowering may have been due to unusually cool temperatures and above-average rainfall during June, July, and August or the trees were in an "off" year of alternate bearing. Weather records indicated that 2004 was the coolest summer on record since 1950 in Missouri.

Another study was conducted to screen thinning hormones currently labeled for fruit and pistachio trees to determine if secondary flowers on Chinese chestnuts can be chemically removed. Chemicals screened included NAA, Accel, and Sevin, alone and

combinations of the materials. Treatments were applied on August 2 when secondary catkins when observed. The number of primary and secondary burs per shoot was recorded, as well as nut number and weight. In 2006, there was a high rate of drop of all 2º burs in many of the treatments by Aug. 30, perhaps due high temperatures (4 days > 37ÿC) that occurred within one week of treatment. However, two of the hormone treatments increased the rate of drop by two weeks after application as compared to the untreated control. Nut number and weight did not differ among treatments at harvest. Treatments were repeated in 2007. Data are currently being recorded from this experiment. As these studies progress, the search continues for the "ideal" cultivar that produces an optimal yield of large, primary nuts each year with little or no secondary flowering.

Fertilizer applications affect on foliar nitrogen, chlorophyll, shoot growth and yield of 'Qing' Chinese chestnut. Three different fertilizer treatments were applied in 2005 and 2006 at equivalent annual rates of nitrogen to determine their effect on growth and yield of 'Qing' Chinese chestnut trees. Chlorophyll meter readings were also obtained to determine their relationship to foliar nitrogen. The fertilizer treatments were 1) 150 kg N/ha in the form of slow–release fertilizer (Pro-Grow) applied on 1 Mar.; 2) 75 kg N/ha in the form of NH_4NO_3 applied on 1 Apr. and on 15 June; and 3) 75 kg N/ha applied on 1 Apr., 30 kg N/ha applied on 15 June, and 45 kg N/ha applied on 10 Oct. all in the form of NH_4NO_3. 'Qing' trees treated with two applications of NH_4NO_3 resulted in greater yield and less terminal shoot growth than other fertilizer treatments in 2005. In 2006, yield was similar, but nut growth may have been influenced by adverse weather conditions. The SPAD-502 chlorophyll meter readings had the strongest relationship to foliar nitrogen when mid-shoot leaves from current season's growth were sampled in mid-June in both years. Thus, the SPAD meter is a promising tool for the rapid assessment of foliar nitrogen of 'Qing' Chinese chestnut trees and for the diagnosis of nitrogen deficiency.

<u>OBJECTIVE 2</u>. To evaluate and integrate multiple approaches for the biological control of the chestnut blight fungus and other pathogens and pests that threaten chestnut, by investigating host/pathogen/parasite relationships from the molecular to the ecological level.

Bill Powell—SUNY-ESF

The American chestnut research and restoration project. Powell presented work that has been done in conjunction with Chuck Maynard (SUNY-ESF) and Scott Merkle (University of Georgia) along with many graduate students and post-docs. Last year, Powell reported that they have two transgenic chestnuts in the field—the first transgenic chestnuts ever outplanted. On June 7, 2006, the 'Wirsig' variety was planted in honor of Stan and Arlene Wirsig. Getting these trees out into the field is proof of the transformation concept. As of 2007, only one of the two trees survived the winter. The survivor is now about 0.5 m tall and has a bushy form. They want to see if the genes are stably expressed after two growing seasons. Powell showed slides of the 'Wirsig' tree and a control tree. Using a GFP marker gene, the 'Wirsig' tree exhibits green fluorescence under UV light. A gel from a RT-PCR showed a band of the expected size of the oxalate oxidase gene. Vascular tissues show low levels of oxalate oxidase. Since

the one surviving tree from 2006, they have planted 16 more this spring; 13 are the 'Wirsig' variety; some are nut grafts. All trees so far have been southern tree backgrounds; they may not do well in Syracuse, NY. They are now beginning to get some trees that are a northern background. As of August 2007, there are 15 surviving transgenic American chestnut trees with putative resistance in the field. Given that it takes 18-24 months to produce transgenic chestnut plants, they are starting a whole new set of trees through the pipeline. They are doing things a bit differently now; they are doing co-transformations—transforming with more than one vector. They are using co-transformation to separate the GFP protein from the gene they want to enhance resistance with. That allows them to still use GFP to follows transformations In the pipeline are the following:

7 co-transformation events embryonic (pGFP +REV) about halfway through the process

- Five transgenic events with pTACF3--AN-2X1, AN-2X2, AN-2X3, AN-2X4, AN-2X5 (WB275-27 with pTACF3, a vascular promoter driving the oxalate oxidase gene)
- JM-1E1 (Pond1-1 with pTACF6, ACS2-ESF39 antimicrobial peptide)
- JM-4E2 (Ellis#1 with pTACF6, ACS2-ESF39 antimicrobial peptide)

There are five additional events that are questionable, but they might become embryonic.

From the embryos, Merkle germinates his embyos. Powell's group develops shoots from the embyos and then multiplies them as shoot cultures to get lots of clones. Also in the early stages of pipeline are: Pond1-1 and 30015-21 transformed with pGFP and p35S-CNO (constitutively expressed chitinase + oxalate oxidase). Next transformations: Pond1-1, Ellis#1, and 30015-2 transformed with pTACF7 (VspB-OxO + ACS2-ESF39).

Powell indicated that their procedure for getting shoot cultures is very good. The problem they have is getting from shoots to whole plants, the process of acclimatization. They have been acclimating their trees in a very old growth chamber. They had more than 500 rooted plantlets but they only have 18 for field planting because they were lost in the growth chamber phase. They are getting new growth chambers and their goal is to have100 transgenic tress to test in the field by 2008 and 1000 in three years. These new growth chambers will control light, humidity and CO_2 concentration. They hope the new chambers will greatly enhance their survival rate.

Nut grafting using tissue culture shoots. They cut open a nut and then very succulent shoots are wedge-cut and the shoot is placed into the open slit in the nut. The nuts are placed in a chamber with moist peat moss and within a few weeks, roots begin to develop from the grafted nut. They have a small grant to try and optimize this procedure.

Subtraction cDNA library. They made a library from resistant Chinese chestnut. Using canker material, they took cDNAs and subtracted out sequences from American chestnut. This removes any sequences that are in common and enhances uniquely expressed genes in resistant chestnuts. John Carlson's lab sequenced a few plates of these-about 150 clones. Several cDNA/gene matches were identified, but a very interesting one was very similar to a diphenol oxidase/laccase gene (10 out of 150 clones). Diphenol oxidase is involved in the oxidation of phenolic compounds and is associated with wound healing, lignification, wood rotting, substrate degradation and detoxification. Diphenol oxidase can be competitively inhibited by oxalic acid.

Competitive inhibition mechanisms match the intermediate resistance seen in Chinese x American chestnut crosses, therefore it is a possible candidate for resistance enhancing gene.

Transgenic elms. A question arose about the public's perception of transgenic trees. There are transgenic elms planted at the Moon Library on the campus of SUNY-ESF. They are growing rapidly—over six feet in a year. A security camera is positioned to photograph any vandalism, but to date, there has been no problem.

Paintball experiment. They have an American chestnut stand that was planted in the 1990s and they are attempting to keep those trees alive. They are using mycelium-filled paintballs to deliver hypovirulent isolates to high cankers. There is a lot of velocity on the paintballs. They will see how effective the treatment is next spring.

Abdelali Barakat—Penn State University, Schatz visiting scientist

Selection for genomic DNA content among blight resistant backcross progeny. This work is being conducted by Kelly Deitrick who conducted a practical application of the dot blot genomic DNA content characterization technique. Deitrick screened 400 trees from TACF's third backcross generation to date, ranking each 1 to 5 for their relative amount of American versus Chinese chestnut genome content. The results have been sent to Hebard to help him select trees for further crosses.

Assembly of BAC clone contigs for the two major blight-resistant loci in *Castanea mollissima*. This work was done in conjunction with Albert Abbott and Laura Georgi at Clemson University. The goal of this work is to prepare a set of BAC clones that covers the two blight resistance loci from *Castanea mollissima* for cloning and sequencing of the blight resistance genes. The steps of this project include:

- Add more DNA markers at the Cbr-1 and Cbr-2 loci (DiLorento and Deitrick)
- Increase the depth of the BAC libraries (Clemson University Genomics Institute)
- Identify set of BAC clones that cover the QTL (Georgi)
- Sequence the BAC clones

Bulk segregant analysis was used to identify 17 RAPD DNA markers that distinguish blight-resistant and blight-sensitive trees around the Cbr-1 and Cbr-2 loci. They found 17 new RAPD markers isolated by BSA; eight were associated with sensitivity to blight and nine were associated with resistance to the blight. All 17 markers were cloned and sequenced to convert into SCARS or PCR-RFLP markers. They are in the process of conducting segregation analyses with these markers in the F2 family in which the genetic linkage map and QTL were mapped previously by Kubisiak. This will determine if the new DNA markers map to the same chromosome positions as the two resistance loci. Sicso ran their data for the first two markers analyzed by Deitrick and found that they do map to the Cbr-1 locus. Georgi has isolated clones from her Chinese chestnut BAC libraries by hybridization to the DNA markers from the original QTL map. Georgi has obtained sequence from the ends of these clones to help order their positions in the QTL. Georgi conducted overgo hybridization of the Chinese chestnut BAC library with the 2 QTL markers CD175 and CD145. The BAC contig order was 101, 199, 154, 160, 147, 205. The end sequences revealed interesting gene sequences and they look forward to full sequencing of the BACs. Georgi will screen the BAC libraries with the BSA markers that Deitrick confirms by segregation to be at the resistance QTL. This will provide a

good starting point for the physical-mapping of the resistance QTLs by the Fagaceae Genomics Project.

Fagaceae project. The goal of this project is to create deep sequence databases for genes expressed in chestnuts, oaks and beech.

Objectives:

- Collect samples
- Prepare cDNAs for sequencing
- Conduct 454 pyrosequencing
- Help with bioinformatics and phylogenetics

Applications:

- Markers for physical and genetic mapping
- Gene tags for functional and comparative genomics
- Discovery of genes for blight resistance

cDNAs that were prepared for 454 sequencing include the following:

1. American Chestnut canker
2. Chinese Chestnut canker
3. Chinese Chestnut 'Mahogany' healthy stem cDNA
4. Chinese Chestnut 'Nanking' healthy stem cDNA
5. American Chestnut healthy stem tree 1 (AC 4T7 stem 1)
6. American Chestnut healthy stem tree 2 (AC 1T6 stem 2)
7. American Chestnut whole plant pool tree 1 (AC 477)
8. American Chestnut whole plant pool tree 2 (AC 1T6)
9. Chinese Chestnut whole plant pool tree 1 (CC BX316)
10. Chinese Chestnut whole plant pool tree 2 (CC GT119)
11. Red Oak-above ground cDNA, trees 1 and 2
12. Red Oak-below ground (root) cDNA, trees 1 and 2
13. Red Oak-above ground cDNA, trees 1 and 2
14. 12. Red Oak-below ground (root) cDNAs trees 1 and 2
15. American beech, whole plant pool tree 1, healthy
16. American beech, whole plant pool tree 2, diseased

The 454 sequence summary is as follows:

Species	Targets	To Date
Chinese Chestnut	145 Mb	172 Mb
American Chestnut	115 Mb	13.4 Mb
Northern Red Oak	70 Mb	27.5 Mb
White Oak	70 Mb	20.9 Mb
American Beech	70 Mb	-----
Total:	470 Mb	233.8 Mb

The bulk of sequencing to date has been on Chinese chestnut cDNA for which they now have 172 M bases of new sequence that has been assembled into approximately 65,000 transcript contigs, each of which have been putatively annotated at NC State University using the BLASTx alignment tool. Their sequence targets for other Fagaceae species are

115 Mb for American chestnut, Chinese chestnut, 70 Mb for northern Red oak, 70 Mb for white oak and 70 Mb for American beech. All of the sequence data from this project will be available at the Genomic Tool Devleopment for the Fagaceae project website hosted by Clemson University Genomics Institute at: www.genome.clemson.edu/projects/fagaceae/. The transcript contigs also will be submitted to GenBank for public access after the final sequence assembly for each species.

Preliminary analyses of microRNAs in cDNAs from *Castanea mollissima* and *Castanea dentata*. MicroRNAs (miRNA) are single-stranded RNA molecules of about 21-23 nucleotides in length regulating gene expression. miRNAs are encoded by genes that are transcribed from DNA but not translated into protein (non-coding RNA). Materials used in the experiment include: American canker and Chinese canker RNA; cDNA library constructed using random primers; and, sequencing of one 454 plate from each sample. PSU's approach for identifying conserved miRNAs is as follows:

1) Search for know miRNAs in 454 contigs
2) Filter sequences with significant hits
3) Blast sequences against miRNA precursors
4) Identify true miRNAs

If sequences are short, try to get flanking sequences. If sequences are long, fold sequence and check secondary structure.

Their approach for identifying nonconserved miRNAs is a bit different from conserved miRNAs.

1) Fold all contigs having no similarity with *Arabidopsis* genes
2) Check secondary structure using miRCheck
3) Check conservation in other species.
4) Chestnut specific miRNAs

Their perspectives on miRNA studies include:

- Continue analyses on 454 sequences to identify non-conserved miRNAs specific to chestnut.
- Clone sRNAs from chestnut and oak and analyze the sRNAome in this two species
- Identify and validate targets of some of newly identified miRNAs.
- Analyze the expression profile of miRNAs controlling the expression of some resistance genes in healthy and canker American chestnut tissues

PA-TACF and PSU chestnut breeding project. This work is conducted by Sara Fitzsimmons. Over 18,000 trees have been planted; survival varies with site. About 35% of planted progeny at each orchard have been inoculated. About 13% of the inoculated BC3 progeny were selected for further breeding. The F1s at Penn State's Stone Valley Research Forest (with John Carlson's lab for genetic mapping study) is looking at a cross of a large American Chestnut female parent tree ("Alex-R") with pollen from *C. mollissima cv.* Mahogany from Sandy Anagnostakis. About 130 seedlings have been planted between 2004-06. The open pollinated F1 families planted at Stokes State Forest in New Jersey include three open pollinated Chinese chestnut families, ~245 seedlings each planted in 2006. They will be inoculated in 2009 for use in genetic association studies by Carlson's lab.

Shawn Kenaley—West Virginia University

Kenaley, a Ph.D. student, is evaluating virulent and hypovirulent inoculum of *C. parasitica* to initiate infection and spermatize virulent cankers. There were two objectives in his study were: (1) evaluate the ability of virulent (V), cytoplasmic hypovirulent (CH) and transgenic hypovirulent (TG) inoculum to initiate infections, and determine whether a relationship exists among season of inoculation (spring, summer, and fall), infection, and canker expansion; and, (2) determine the effectiveness of virulent and hypovirulent inoculum to spermatize virulent cankers.

Objective 1 methods included periodic field inoculations. Inoculations were performed at eight-week intervals from May-September 2006. Nine inoculum types were employed: (1) TG-ascospores; (2) V-ascospores; (3) CH-conidia; (4) TG-conidia; (5) V-conidia; (6) CH-mycelium; (7) TG-mycelium; (8) V-mycelium; and, (9) control (0.1% peptone). A total of 675 inoculations (75 per treatment; 25 per interval) were performed, and the incidence of infection and linear growth of resultant cankers was recorded. All cankers were sampled on their 1-yr. anniversary. Results of May and July 2006 inoculations are presented. Results of his work are as follows:

Ascospore inoculations: After 1 yr., no significant difference in the incidence of infection was evident between V- and TG-ascospore treatments for May and July 2006 inoculations. For May 2006, TG- and V-ascospore treatments resulted in 6 and 5 infections, respectively. Few infections (n=3) were observed for both treatments for inoculations performed in July 2006. No significant interactions were observed between strain and month of inoculation or infections by tree. Month of inoculation did not influence the growth of cankers initiated by TG- or V-ascospores, and no strain-month interaction was detected. However, the mean linear growth for TG- and V-ascospores was significantly different after one year. Although number of infections was limited for both strains by month, treatment inoculum was recovered from sites exhibiting no canker development.

Conidial inoculations: Comparing infection by strain-spore type after 1 yr., inoculations performed with V-conidia resulted in the greatest number of infections, 52% (13/33) of the observed infections for May and 24% (6/13) for July 2006. The observed infection by strain and month were significantly different. The infectivity of CH-and TG-conidia was significantly reduced compared to V-conidia for both months of inoculation. The mean linear growth of cankers initiated by conidia was significantly different by inoculum type. No difference in growth by month of inoculation or strain-month interactions was observed. Canker growth for V-conidia initiated cankers was significantly greater than CH-and TG-conidia initiated cankers, and no difference was observed between the linear growth of CH and TG cankers.

Mycelial inoculations: The greatest number of infections by strain was achieved using mycelium. The number of observed infections for mycelial inoculations was significantly different by strain. For May 2006 performed inoculations, V-mycelium was 100% effective (25/25 inoculations) in initiating infection, followed by CH-mycelium (56%; 14/25 inoculations) and TG-mycelium (40%; 10/25 inoculations). The incidence of infection by strain was reduced significantly for inoculations performed in July 2006. The growth of mycelial-initiated cankers was different by strain. The growth of V-

mycelial cankers was significantly greater than CH- and TG initiated cankers. No difference in growth was observed between CH- and TG - mycelium.

Materials and methods for objective 2 for spermatization testing included the utilization of eight treatments as possible spermatia: (1) TG-ascospores; (2) V-ascospores; (3) CH-conidia; (4) TG-conidia; (5) V-conidia; (6) CH-mycelium; (7) TG-mycelium; and, (8) V-mycelium. In May 2006, fifteen cankers per treatment were established using EP155, and treatment inoculum was applied three times between June 2006 and the first week of August 2006. Outcrossing to treatment was observed for CH-conidia (11/18 perithecia), TG-conidia (8/35 perithecia), TG-ascospores (8/37 perithecia), and V-conidia (8/40 perithecia). V-ascospores and mycelium for all strains failed to spermatize artificially-initiated cankers. Results of the TG-ascospore treatment are being examined to determine whether TG-ascospores or TG-conidia were responsible for the observed outcome. Conidia for all strains were equally effective at spermatizing.

Mark Double—West Virginia University

Transgenic *C. parasitica* study. Double reported on the biological control potential of *Cryphonectria parasitica* strains containing an infectious cDNA copy of the hypovirus CHV1-Euro7. This work was initiated by Bill Rittenour and is conducted in cooperation with D.L. Nuss, University of Maryland Biotechology Institute. This study 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 were employed to compare transgenic hypovirulent (TG), cytoplasmic hypovirulent (CH), and virulent (V) strains. To produce ascospore inoculum, naturally occurring and artificially established punch-initiated (PI) cankers were spermatized by painting cankers three times each summer with a conidial mixture containing MAT-1 and MAT-2 mating types of the appropriate treatment strain (TG, CH, or V). Conidial inoculum was produced by inoculating scratch wounds made to the bark with a mycelial slurry of the appropriate treatment strain (TG, CH, or V). Non-treated trap (T) trees were left to monitor natural canker development as well as hypovirus spread. Tree condition and natural canker establishment were assessed for all trees in August of each year. As of August 2007, there were 111 natural cankers in TG plots, 60 in CH plots, and 48 in V plots. Cankers were sampled, when detected and each November, to determine the hypovirus infection status of the thallus. Although the purpose of the spermatization treatment was to produce ascospores, many treated cankers also acquired hypovirus from the treatment inoculum and have begun to produce callus.

Some significant findings this past year include the increased detection of hypoviruses, both in the canker thallus and perithecial outcrossing over the previous two years. Hypoviruses were detected in cankers on trap trees for the first time, both in the thallus and as spermatia. Transgenic inoculum was detected in not only TG plots but also in CH and V plots. The types of ascospores produced were assessed by pigmentation and morphology. Twenty-four thousand individual ascospore colonies were examined from 988 individual perithecia in 2006. Hypovirulent ascospore isolates were collected from 76% of the spermatized cankers in TG plots. From those cankers, 47% of the perithecia yielded trangenic ascospores.

Backcross orchard for assessment of host resistance combined with hypovirulence. This experiment is conducted cooperatively with Fred Hebard and Bob Paris of The American Chestnut Foundation. Six replicate plots containing 150 trees each were established at the Plant and Soil Sciences Farm in Morgantown, WV in the spring of 2005 for the purpose of assessing the interaction of host resistance and virulent/hypovirulent strains of *Cryphonectria parasitica*. Backcross lines along with pure American, Chinese and European chestnuts were included in plantings. Raccoon predation of the seed was very heavy in 2005; plots were replanted in the April 2006. The raccoon population was reduced by live trapping and although predation still occurred, the impact was less severe. As of September 2006, 30% of seedlings had survived. The plots were replanted in March 2007 and all 900 planting sites were caged with fencing. As of August 2007, 48% of the planted seedlings were growing.

William MacDonald—West Virginia University

Introduction of hypoviruses at West Salem, Wisconsin. This work is conducted in cooperation with J. Cummings-Carlson, Wisconsin DNR; 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; from 1998-2002 hypoviruse introduction was halted. Beginning in 2003 through 2007, hypoviruses were reintroduced via two methods: (1) punch treatment of individual cankers; and (2) wounding of trees by inoculating scratch wounds to the bark surface to create reservoirs of inoculum. In 2001, twelve permanent plots were established in three regions of the stand (Disease Center; Front and Beyond the Front). Half the plots in each region were punch treated and the remaining half were scratch-treated. Approximately 25% of the trees in each plot were untreated.

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

Each spring all cankers are sampled and tree health evaluated. Results from 2006-07 are as follows:

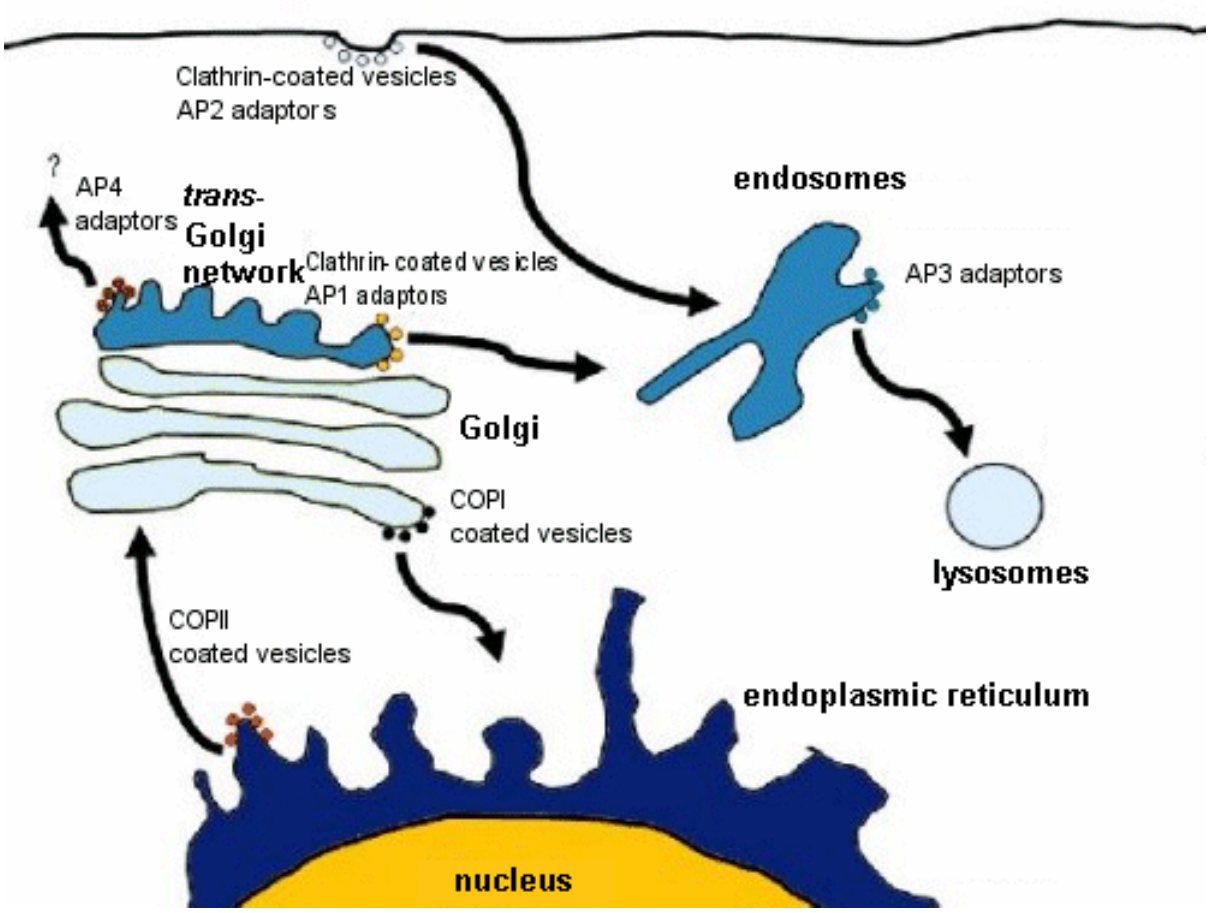
- A total of 2371 cankers have been detected in the 12 plots since 1992; 712 cankers on living trees were sampled in May 2007; 212 were newly discovered.
- The number of infected trees has remained relatively constant in the Disease Center plots (92-96%) between 2001 and 2007. In contrast, the number of infected trees has risen sharply in the Disease Front plots (29% to 85%) and Beyond the Front plots (11% to 78%).
- Reservoirs of hypovirulent inoculum, provided by scratch-wounding areas of the bark, were not as effective when compared to punch-treated cankers in disseminating hypovirus inoculum to new infections.
- Comparing data from 2001 through 2006, all cankers, regardless of whether on treated or non-treated trees, increase the components of hypovirus and non *C. parasitica* while the amount of virulent *C. parasitica* has dropped.

- Vegetative compatibility testing continues. WS-1 continues to be the dominant vc type in the stand; its recovery rate has remained steady for three years at 87%. WS-2 and WS-3 were found at rates of 1% and 8%, respectively.
- Mortality in the, Disease Center, Front and Beyond the Front is 44%, 25% and 21%, respectively.

Hypovirus treatment appears to play a role in tree longevity. Fifty-four percent of the trees in the Disease Center that initially were treated from 1992-1997 were alive in 2007. Only 45% of the trees infected between 1998-2002, in the absence of hypovirus treatment, remain alive.

Debora Jacob-Wilk—UC-Davis

Silencing of *Kex2* and its effect on virulence of *C. parasitica*. The hypovirus causes vesicle accumulation in *C. parasitica* and they want to characterize these vesicles. She found that four CHV1 markers accumulate in the microsomal fraction: dsRNA, polymerase, helicase, p29 protease. These viral markers co-fractionate with a peak in Kex2 activity and with AP-1ÿ, two trans-golgi network (TGN) markers. She wanted to characterize the vesicles to see what fungal proteins were being affected. The fungus down regulates several genes: cryparin, laccase and pheromone. Kex2 is a protease that localizes to the trans-golgi. ER marker KDEL and intermediate compartment ÿ-COP marker migrated to upper fractions in the gradient. This shows a clear, distinct localization between the ER and intermediate compartment and the viral vesicles and the TGN. The mycovirus CHV1 elements cofractionate with the fungal host trans-golgi membranes. P29 is membrane associated and it is predominantly associated with the cytoplasmic face of the vesicles. A diagram of the secretory pathway follows:



Kex2 protease: Kex2 is a transmembrane, Ca^{2+} dependent serine protease of the Sutilisin superfamily with a primary specific cleavage at the carboxyl side of Lys-Arg and Arg-Arg sequences within polypeptide precursors.
Serine proteinases of the kexin/subtilisin superfamily activate precursor forms of many exported eukaryotic proteins and play crucial roles in a large and varied set of biological processes in animals, plants and fungi.
They participate in both the constitutive and regulated secretory pa thways, with substrates including hormones, neuropeptides, serum proteins and surface receptors. Kexins also mediate the maturation of viral proteins, bacterial toxins, pheromones, zymogenes of secreted proteases, lipases, polysaccharide-degrading enzymes, etc.

Jacob-Wilk cloned the Kex2 gene and found it to be analogous to Kexins in other fungi such as *Neurospora crassa* (63%-76%) and *Magnaporthe grisea* (59%-72%). She shows high domain conservation. She tried to knock out the Kex2 gene but after screening more than 1000 colonies, she was unable to obtain a deletion so she pursued gene silencing. To do this, she constructed a vector using Nuss ' pCPXHY1. She transformed Ep67, a wild-type isolate of *C. parasitica* and selected for differing degrees of silencing. She showed two selected transformants, Mut-G and Mut-P. Showing southern blots, Jacob-Wilk demonstrated that there was integration of the silencing vector into the genome and

the endogenous locus was not affected. She also produced antibodies against expressed Kex2 protein in *E. coli*. Using western blots of the wild type compared to the silenced transformants, there was constitutive expression of EP67 and Mut-P and there was no detection of Kex2 in Mut-G. In terms of relative transcript levels, there was 70% difference between the control and the mutants. They proved that the silenced genes they selected for were indeed silenced. She went on to check virulence and used apples for this detection. Ep67 produced a large lesion while Mut-P produced a small lesion and Mut-G produced almost no lesion. Put together with growth rate of the fungi on agar media, she showed that the fungi grow well so the mutants are not the result of generalized debilitation of the fungus, but due to silencing. She checked virulence on excised stem pieces and the data is as follows:

Strains and mutants	**Mean canker area (mm^2)**	**±SD**
Mock	6550	836
EP67	6767	941
Mut-P	3727	1312
Mut-G	183	288

Kex silencing also had an effect on mating as shown in the following table.

Female strain	**Male strain**	**Plates with Perithecia**	**Ascospores**
EP155	EP67	5/6	+
EP155	Mock	6/6	+
EP155	Mut-P	3/6	-
EP155	Mut-G	3/6	-
EP67	EP155	6/7	+
Mock	EP155	7/7	+
Mut-P	EP155	7/7	+
Mut-G	EP155	3/7	-

When she introduced the virus to the silenced transformants, western blots indicated that the virus suppressed gene silencing.

To summarize, results indicate that Kex2 is an essential factor in the virulence of *C. parasitica*, either directly or indirectly through the processing of pro-proteins involved in the virulence process, such as host recognition, attachment or cell wall degradation. Several Kex2 substrates known to be virulence factors include: secreted aspartyl proteinases, exoglucanase (Xog1p), adhesins (Hwp1p, Cryparin) and pheromone (ÿ-factor). Other putative Kex2 substrates include several adhesions, cell wall proteins and hydrolases.

Her hypotheses are:

- The virus affects virulence through the disruption of the secretory pathway. This inhibition would cause TGN-vesicles in transit and their cargo to accumulate in the cytosol not reaching their target membranes or reaching themin a delayed manner.
- At the same time, the vesicles that accumulate not only are unable to reach their destination, but are also unable to recycle back. Therefore, and even though Kex2

is present and active in the hypovirulent strain, it is sequestered from its original processing site as are its wide array of substrates from their target membranes.
- It is proposed that these vesicles are unable to fuse with target membranes either due to conformational or functional interference of the proteins involved by viral proteins or due to the aggregation of these vesicles among themselves.

Don Nuss—University of Maryland Biotechnology Institute, Shady Grove

Community Sequencing Program update. The project title, 'Genome sequencing of the chestnut blight fungus *Cryphonectria parasitica*' was submitted in March, 2006 and approved June, 2006. Donald L. Nuss, University of Maryland Biotechnology Institute is the project proposer. Other proposers are Alice C. L. Churchill and Michael G. Milgroom, Cornell University. Other participants include: John Carlson, Pennsylvania State University, USA; Baoshan Chen, Guangxi University, PR China; Angus Dawe, New Mexico State University, USA; Bradley Hillman, Rutgers University, USA; Dae-Hyuk Kim, Chonbuk National University, South Korea; Thomas Kubisiak, USDA Forest Service, USA; Myron Smith, Carleton University, Canada; Neal Van Alfen, University of California, Davis, USA; and, Michael Wingfield, University of Pretoria, South Africa.

What will JGI/DOE CSP do?
- Provide a 8-10X shotgun sequence of 40Mb genome.
- Assemble genome sequence.
- Generate 20,000 expressed sequence tags (ESTs) for annotation effort.
- Automated and directed annotation.
- Provide web-accessible database with tools for mining and comparative genome studies
- Sponsor a jamboree for manual annotation.

DNA and RNA samples were sent early enough in the year that we were first in line for the process. The timeframe for various activities is listed in the following table.

Library construction and quality control	3-6 months
Sequencing	3-5 days
QC and assembly	3-6 months
Machine annotation	3-6 months
Jamboree, manual annotation	3-6 days
Public release of draft sequence	
Finishing-filling in the gaps	6-12 months
Additional annotations	3-6 months
Final release	
Continuing annotations	5-10 years

How do we get there:
- Automated annotation will identify likely reading frames.
- EST sequences will confirm expression of putative genes and will indicate intro/exon boundaries; they will provide an excellent source.
- The computer will make mistakes, so manual verification of computer predictions is essential.

- A group of annotators will work on genes of interest.
- An annotation jamboree is excellent and should be scheduled after a period of manual annotation.

At the jamboree:

- Specific classes of genes are assigned to annotators based on interest and experience
- Annotators are allowed access to the sequence prior to the jamboree (3-6 months)
- Manual improvement are made to the computer predictions
- Involved individuals meet for 2-5 days to present and discuss results
- Everyone is required to make a presentation on their findings so that all participate.
- Annotation is a continuing process.

What is included in this process—many of the following:

Basal metabolism	Gene clusters
Carbon metabolism	Gene families
Cell communication	Glycosylation pathways
Cell wall metabolism	Heatshock proteins
Chromatin	Incompatibility genes
Cytoskeleton	Iron metabolism
DNA polymerases	Microsatellites
Energy production	Mitochondrial genome
Fatty acid biosynthesis	NRPSs
P450s	Silencing pathways
Photoreceptors	Sterol biosynthesis
Polyketide synthases	Strees genes
Proteases & inhibitors	Terpene synthases
RNAi proteins	Transcription factors
Secreted proteins	Transmembrane proteins
Sexual development	Transposons
Signal transduction	

What will be done with the sequence? Some of questions that the genome sequence will help us address.

- Secondary metabolism
- Vegetative incompatibility
- Response of *C. parasitica* to hypovirus
- Comparative fungal genomics

An annotation committee has been set up. It includes: Nuss, Churchill, Milgroom, Hillman and Dawe. Assignments on various gene categories have been made. The following individuals are not responsible for, but are interested in the following categories:

- Secondary metabolite pathways (Churchill)
- Vegetative incompatibility/heterokaryon incompatibility (Hillman, Milgroom)
- Homologues of genes associated with virus replication in yeast (Hillman, Nuss)

- Mating type gene clusters (Crouch, Milgroom)
- Microsatellites, transposons (Crouch, Milgroom)
- RNA silencing genes (Choi, Nuss)
- Autophagy-associated genes (Choi)
- Apoptic pathway (Dawe, Hillman, Milgroom)
- Proteosome pathway (Choi)
- N-mynistyltransferase (Hillman)
- G-protein couple receptors (Dawe)
- Clock function and clock responsive genes (Dawe)
- Oligopeptide transporters (Dawe)

Nuss indicated that he will seek individuals with expertise beyond what the organizing committee has. Some categories where additional expertise is needed are as follows:

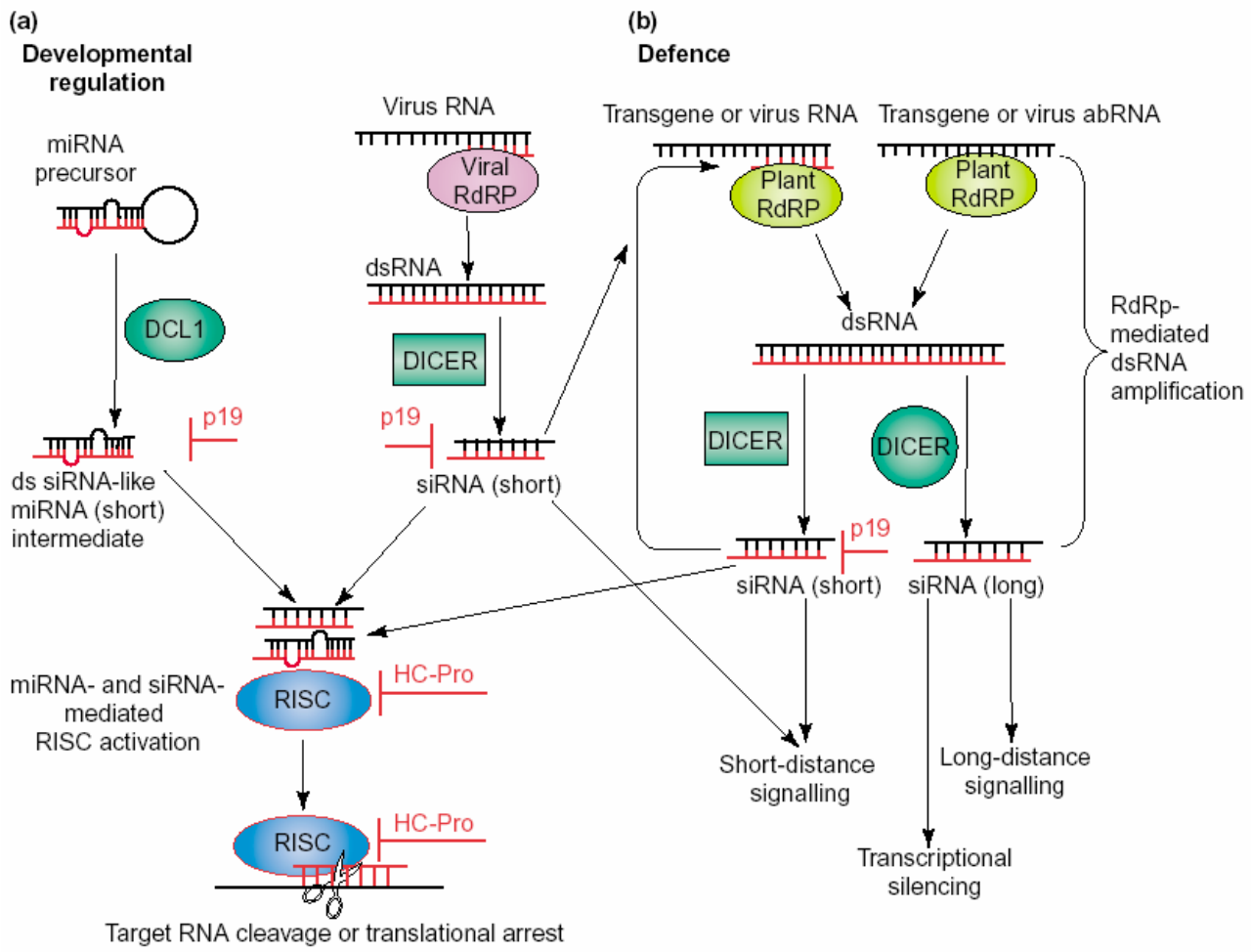
- CK1, CK2, PKC, PKA, kinases
- Extracellular digestion
- Secreted proteins
- Transposon factors
- Stress response
- Other signal transduction pathways
- Homologues to published pathogenicity genes
- Whole genome comparison
- Proteins unique to *C. parasitica*
- Asexual and sexual sporulation genes

Prior to the NE-1015 meeting, Nuss received report from JGI. Some of the specifics of that report are:

- 20,000 *C. parasitica* ESTs have been sequenced
- 8X sequence of *C. parasitica* genome has been completed
- 8X assembly is ready for September
- Automated annotation should be completed by January 2008 at which time JGI will create a genome portal and provide training for the community to begin manual annotation
- The annotation genome sequence will go public six months following the beginning of manual annotation
- JGI would like to see a publication of results of the genome project

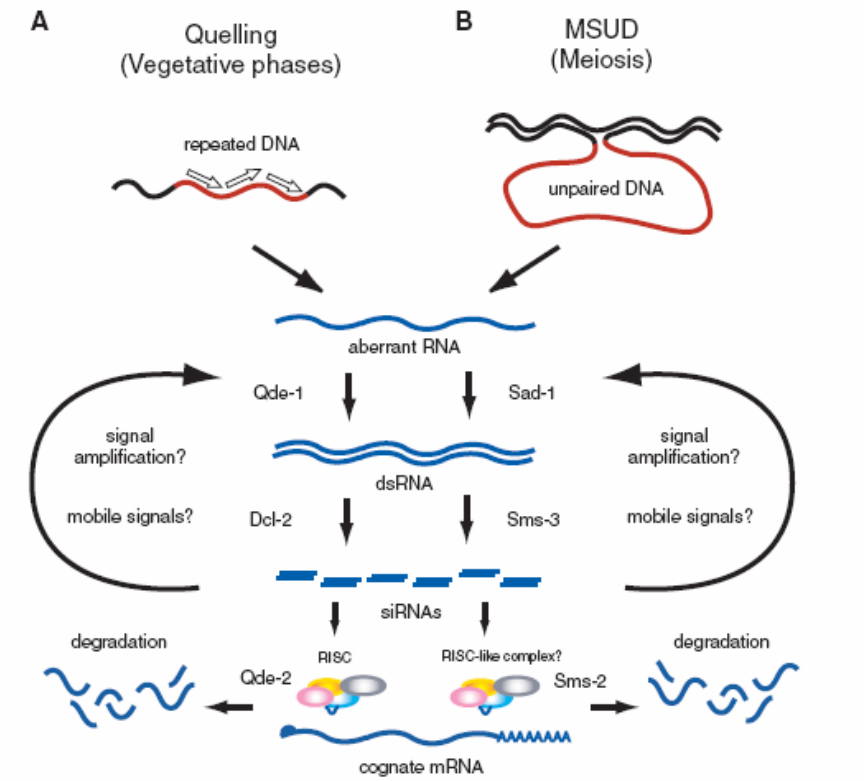
There should be plenty of information to be presented at the 2008 NE-1015 meeting.

RNA silencing as an antiviral defense mechanism in fungi. The chestnut tree puts up defenses when infected by the chestnut blight fungus. The fungus also has defense mechanisms, that hen infected by viruses, alter the ability of the fungus. There is silencing going all at all levels; there is RNA silencing in *C. parasitica* that is an antiviral defense mechanism to infect the plant host. RNA silencing is basically a small RNA (micro RNA--miRNA or small interfering RNA--siRNA)-mediated, sequence specific gene regulation. In plants it is called post-transcriptional gene silencing (PTGS) or if you use viruses as vectors, it is called virus induced gene silencing (VIGS). In animals, it is called RNA interference or RNAi. In fungi, it is called quelling or meiotic silencing by unpaired DNA called MUSD. Nuss showed the following diagram of RNA silencing in plants.



There are many players involved in silencing. miRNA production is involved in developmental regulation in plants and animals, but apparently not in fungi; no miRNAs have yet been found in fungi. Precursors containing hairpin structures are processed by RNAseIII molecules called Dicers. Produced are small dsRNAs or siRNAs that are incorporated into a RISC complex or a RNA-induced silencing complex. That is facilitated by other components of the system called Argonauts. That complex uses one of the stands of the small RNA to target RNA cleavage. These small RNAs are involved in the regulation of gene expression of a lot of key plant and animal genes. These silencing systemsalso respond to viral RNA—either dsRNA or structured regions of the viral RNA and Dicers cut out small RNAs that are incorporated into a RISC complex. In this case, in most instances, the strand that then is used as the guide will recognize the viral RNA from which it was derived, resulting in cleavage and destruction of the RNA which gives rise to the antiviral defense mechanism. This can be amplified because some of the small RNAs can be used as primers by post RNA-dependent RNA polymerases to make more dsRNA substrate for the Dicer to cleave that to small RNAs and that can travel through the plant or increase in concentration to reduce the amount of RNA present.

In fungi, most of the work that has been done on silencing has been done with *Neurospora*.



There are two pathways, however, Nuss is interested in only the quelling or vegetative phase. This pathway recognizes trans-genes for aberrant RNAs usually turning them into dsRNAs by an RNA-dependent RNA polymerase. Again, there is a Dicer that recognizes and cleaves it, incorporating it into a RISC complex resulting in the destruction of cognate mRNAs. This is how you get rid of transposable element RNAs or trans-gene RNAs.

Nothing was known about whether or not RNA silencing could be an antiviral defense mechanism in fungi, as it has been reported to be used in plants and animals. The current view of RNA silencing in fungi is primarily derived from *Neurospora.* However, there are no well characterized mycoviruses available for *N. crassa*. The chestnut blight fungus is closely related phylogenetically to *N. crassa* and has a well established experimental system of mycoviruses and the various roles of RNA silencing in fungi. Thus, *C. parasitica* was chosen to look at RNA silencing as an antiviral defense mechanism. There were some hints that this was the case because p29 had similarities to a suppressor of RNA silencing Potyviruses HC-Pro. Viruses, in general, have evolved the ability to encode suppressors of RNA silencing to defeat the antivirus defense mechanism. So HC-Pro was the first one to be discovered and it has a lot of similarities to p29. One of the first questions to ask is, 'Does p29 act as a suppressor of RNA silencing?'

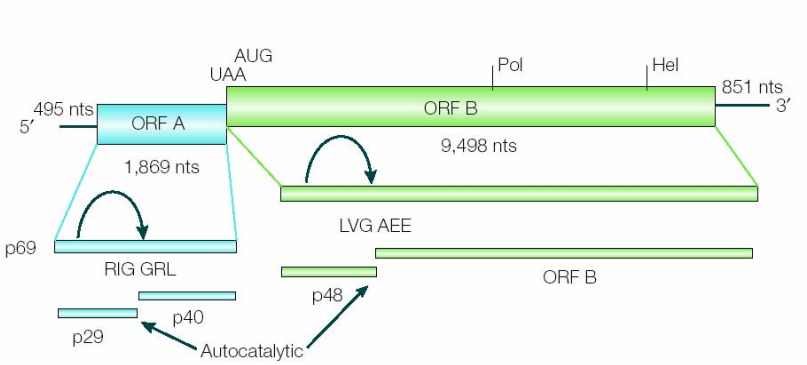
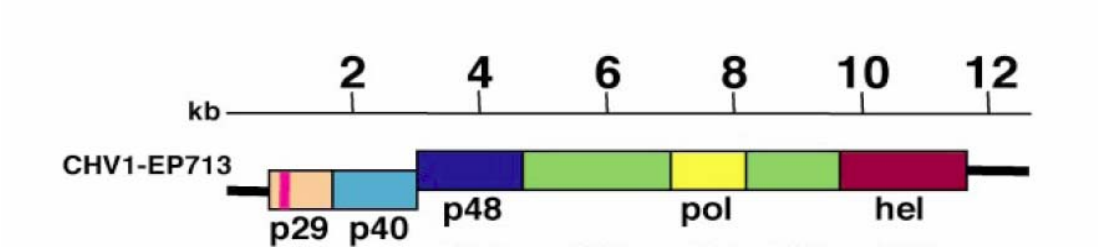
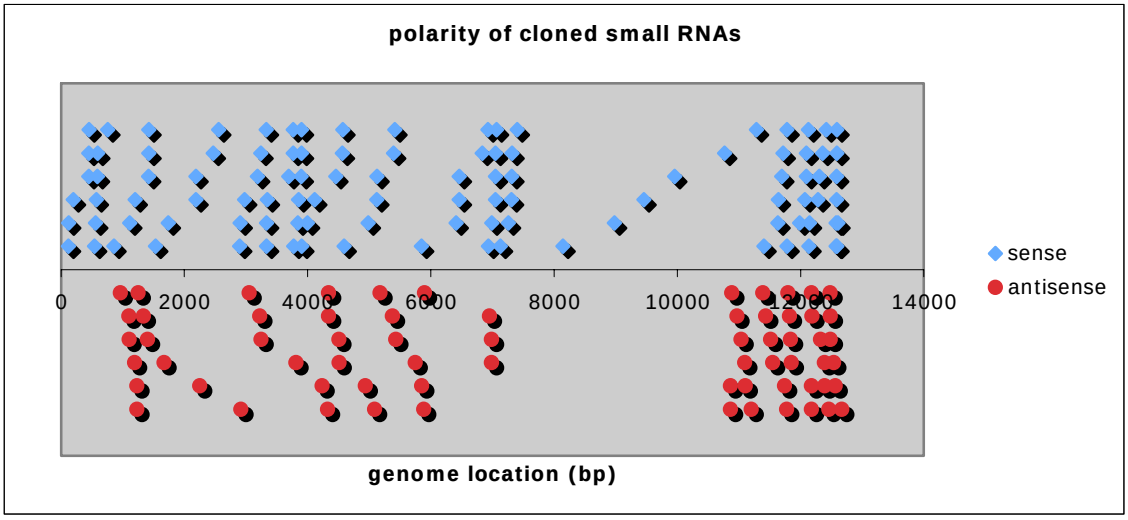


Figure 2 | **Genetic organization and basic expression strategy for prototypic hypovirus CHV1-EP713.** The coding strand RNA of hypovirus CHV1-EP713 consists of 12,712 nucleotides (nts), excluding the poly(A) tail[15]. The 5′ proximal coding domain, ORF A (composed of 622 codons), encodes two polypeptides, p29 and p40 that are released from a polyprotein, p69, by an autoproteolytic event mediated by p29 (arrowed). During translation, cleavage occurs between Gly248 and Gly249 of the target sequence RIG GRL, and is dependent on Cys162 and His215 in the putative p29 catalytic site[58,59]. Expression of ORF B (composed of 3,165 codons) also involves an autoproteolytic event (arrowed) in which a 48-kD polypeptide, p48, is released from the N-terminal portion of the encoded polyprotein. Cleavage, in this case, occurs between Gly418

A RNA hairpin silencing assay was set up. A GFP gene was set up in opposite orientation with an intron in the middle; you can then transform *Cryphonectria* that is already expressing GFP. Because of that hairpin RNA introduction, you end up with a reduction in the expression of the target gene; thus the gfp gene was silenced by hairpin-gfp RNA.

Nuss was able to show that p29 activity suppresses this hairpin RNA silencing process in fungi, giving circumstantial evidence that RNA silencing may serve as an antiviral defense mechanism in fungi. Also, using well-established RNA silencing assays in plants, he was able to show that p29 also suppresses RNA silencing in *Nicotinia benthamiana*. In order to seek direct evidence that RNA silencing may serve as an antiviral defense mechanism, two questions were posed. First, are virus-derived siRNAs produced in mycovirus-infected *C. parasitica* as a result of antiviral RNA silencing? Second, does the disruption of the RNA silencing components in *C. parasitica* alter mycovirus-mediated symptoms and virus accumulation? With regard to the first question, he looked for small RNAs; there are a number of procedures that allow you to score for very small RNAs (in the 20 nt range). He used a probe that represented either the + or – strand of the virus. You can find signal that corresponds to what look like virus-derived small RNAs in the infected strain, CHV1-Ep713. Those bands were cut out. Two-hundred, thirty-four clones were sequenced and 73% corresponded to viral RNA and 27% to other RNA. He looked at the size distribution and the virus-derived peaked at 21nt. He was interested in looking at viral specific—the 21nt matched the sequence of the virus; it can be determined whether it was derived from the + or – strand of the virus. Findings were that 60% came from the + strand and 40% from the – strand. The 5' end of the non-coding region of the + strand has lots of secondary structures—probably substrates by the Dicers then to generate dsRNAs. They have not found any dsRNAs that correspond to the – strand for that region, suggesting that there are considerable structured regions.





In the above graph, there is a lot of clustering; it is not non-random, so there are hot spots for the generation of these vsRNAs. For some reason the area of the helicase/polymerase region is very low in production of vsRNAs. This may be related to the generation of defective RNAs or it may be an area of secondary structures that protects against Dicers. The current view, of generation of these small RNAs for most + strands of plant viruses, is that small RNAs are derived predominantly from highly structured single strand viral RNA and not from double stranded intermediates. Hypoviruses have no coat protein so there is a lot more dsRNA that accumulates in the infected cell.

Pertaining to the second question they posed, 'does the disruption of the RNA silencing components in *C. parasitica* alter mycovirus-mediated symptoms and virus accumulation?' the most direct way is to disrupt part of the RNA silencing pathway. Two dicer-like genes were cloned from *C. parasitica* based on conserved amino acid sequence of *dcl* genes from *N. crassa*, *M. grisea* and *F. graminearum* They knocked out Dicer 1 and Dicer 2 and looked at the effect of virus infection on the expression of Dicer genes. The results were interesting. Looking at Dicer 1 and Dicer 2 transcripts, there were low levels of both in uninfected Ep155. In CHV1-Ep713, the level of Dicer 1 was low but the level of Dicer 2 was 15-20 fold increase. In the Reovirus, MyRv-9B21, infected strain there was also a nice induction (better than 713) of virus accumulation of Dicer 2 but not Dicer 1. In Æp29 infected strain, there was a super induction suggesting that, among the many functions it might play, p29 suppresses the induction of virus-induced Dicer 2 transcript.

There is no phenotypic change as a result of the deletion of the Dicers. Putting hypovirus and reovirus into the Dicer mutants, the CHV1-infected the Dicer 1 look just

like the wild type. With Dicer 2 and the Double Dicer, there is a very severe phenotype with the virus suggesting that there was a disruption of the antiviral defense mechanism. There was a similar scenario for the reovirus, although the debilitation was not quite as severe as with the hypovirus.

Summary points:

- Hypovirus-encoded p29 is a suppressor of RNA silencing (first report for a myovirus)
- *C. parasitica* Dicer-like gene *dcl-2* transcription is induced after mycovirus infection and this induction may be suppressed by p29 (novel mechanism for induction and suppression of RNA silencing?)
- Dicer-like gene *dcl-2* is required for the antiviral defense while *dcl-1* is dispensable (direct evidence for RNA silencing as an antiviral defense mechanism in fungi
- Hypovirus vsRNAs in *C. parasitica* are derived from both plus (60%) and minus (40%) strands of the viral genome. The average size range is 20-22nt (do mycovirus-derived vsRNAs influence fungal host gene expression?)

Bradley Hillman—Rutgers University

Hillman reported on the bacterium *Lysobacter* on which he reported in 2006.

Lysobacter enzymogenes – a fungal-infecting bacterium:

- Gram negative, soil inhabiting bacterium
- Closest relatives: *Sphingomonas, Xanthomonas, Burkholdaria*
- Has Type I, II, III, IV and VI secretion system
- Produces lytic enzymes and antibiotics prolifically
- Enters and likely multiplies in lower eukaryotes: true fungi, stramenopiles (*Phytophthora*), nematodes *C. elegans*), lower plants (*Physcomitrella*)
- Draft sequence completed July 2007 (Kobayashi, Hillman, Lawton, Crouch, Sullivan; TIGR)

The Type III secretion system that he thought might be important for the pathogenesis of fungi appears not to be important. He noticed in the genome sequence project that they have for *Lysobacter* that it also has a Type VI secretion system, a novel section system that was characterized 3-4 years ago. They are focusing on that as something that may be fungal specific. One reason they are interested in this bacterium is that it infects not just fungi but a number of lower Eukaryotes. It does not infect higher plants or higher animals.

The genome annotation was completed this summer; the sequence is about 6.4 Mb. The sequence was machine annotated; Hillman and Crouch assisted with the manual annotation. About 2 of 3 genes that were machine annotated—when they were looked at manually with more stringent set of rules, they unannotated them. The computer annotation was correct, but the stringency was not great enough for TIGR to say this is what it is. Hillman commented that the *Lysobacter* genome will be an interesting genome to work with.

Lysobacter has a very high GC content (around 63%) which makes it very difficult to work with. *Lysobacter* greatly damages Petri dish colonies of *Colletrotrichum* and *Magnaporthe* but there is no damage on *C. parasitica*. They are just as interested in negative interactions as positive interactions. This summer, Hillman's son Tom looked at

the interactions of *Lysobacter* and *C. parasitica* microscopically using a GFP and found that, in fact, *C. parasitica* is attacked by *Lysobacter* at the microscopic level. The bacterium does internalize in the fungus, but at this point, they do not know the extent of damage. With the genomics of *Magnaporthe* and *Lysobacter* known already and with the genomics of *C. parasitica* coming this fall, the genetic tools should allow for a fairly well defined system.

Hillman introduced Jo Anne Crouch, a Ph.D. student in his lab who will finish her dissertation in a few months. Crouch works on *Colletotrichum* but she has a number of side projects. One of these projects deals with *C. parasitica*. His other student, Sara Baxer is working on eastern filbert blight.

Jo Anne Crouch—Rutgers University

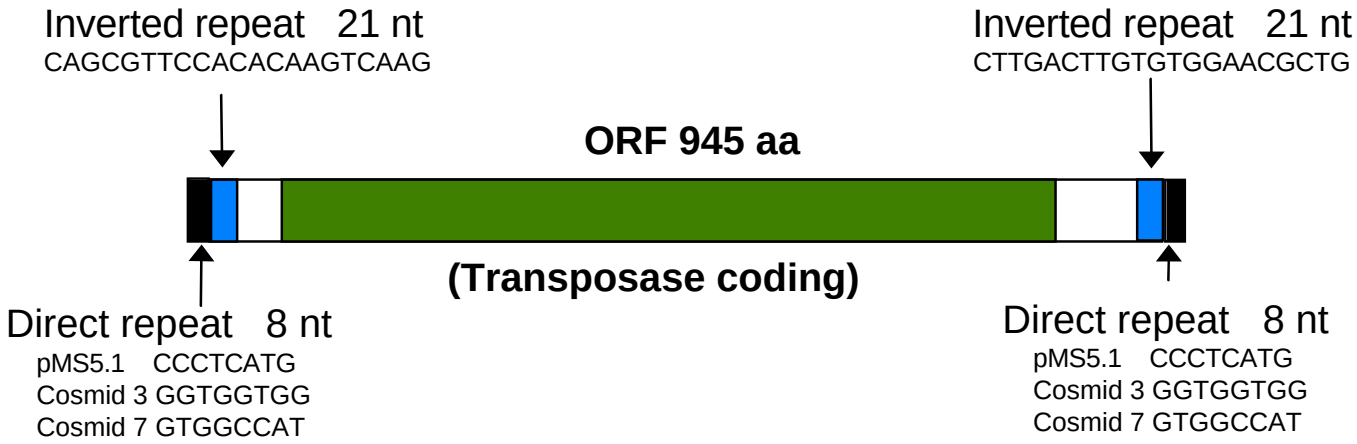
The evolution of extrachromosomal elements in the *Cryphonectria parasitica* genome. Crouch is working on two projects that deal with viruses and transposons. *C. parasitica* has a lot of extrachomosomal elements in the form of dsRNA viruses. One of the best characterized is the CHV1 virus from Europe.

Michael Milgroom has been working on this data set for eight years with respect to where the virus originated and how it moved across Europe. The hypothesis is that CHV1 is native to eastern Asia and moved along with *C. parasitica*. There is data to support this. Early work using RT-PCR was able to define five distinct subgroups. Subsequent sequence analyses showed support for only three groups. Milgroom got Crouch interested in the variability not only in Euorpe but also in Asia. In looking at the *C. parasitica* population in Italy, there are two populations within the Italian subtype 1—a northern and a southern population were differentiated. This indicated that there was restricted migration and divergence due to drift. Carbone and others speculated that the sequences of CHV1 had either rapidly diverged since the introduction or that separate introductions occurred in Italy. The only study reported to date that looked at CHV1 in Asia does not provide insight into the origin of CHV1 into Europe. Partial nucleotide sequences from EP713 and Euro7 were not closely related to any of the sequences from isolates sampled in Japan. Relative to the Japanese CHV1 isolates, EP713 and Euro7 are most similar to a virus sampled from *C. nitschkei*. To test whether CHV1 was introduced to Europe from eastern Asia, and to estimate how many introductions might have occurred, they are conducting phylogeographic analyses of CHV1 from Europe and Asia. They looked at CHV1 from the following populations: *Asia*—15 from Korea; 26 from Japan; 25 from China and *Europe*—4 from France; 1 from Spain; 1 from Germany; and, 31 from Italy. They looked at the two coding regions (ORF A and ORF B) and both have been subject to very strong negative selection pressures. In the three regions they examined (ORF A=279nt; ORF B=474nt and ORF B=1095nt, they found two interesting results. First, the amino acid sequences have evolved into highly conserved regions. Second, recombinations have shaped all three of the regions that they looked at. Strong purifying selection has shaped both coding regions. They used six separate recombination detection methods to identify recombination: calculation of Phi_{theta} in Splitstree, GARD recombination breakpoint method, STRUCTURE, visualization of reticulation using Splitstree, LAMARC and SH likelihood ratio tests of sliding window datasets. There is a great deal of homoplasy caused by recombination in the data set. (Homoplasy is that they are not homologous but they look alike by chance).

There is a great deal of diversification in the Asian population. There is a Korean group which is quite divergent. There were a few things that were unexpected. First, all of the Chinese isolates grouped with the same population as NW Europe. Milgroom believes that European CHV1 may have been reintroduced into China. Second, there is a great deal of recombination occurring between the different subgroups. Using coalescent-based estimates to determine the migration between populations, the data came up in a non-sensical manner. They may not be able to track migration and Milgroom thinks this may have something to do with the fact that these are not very old populations, less than 100 years of history. The data does not allow them to strongly state that CHV1 migrated from Asia to Europe.

Transposable elements. Transposons are extrachromsomal elements that have the ability to jump around in the genome. They can significantly impact the way a host genome evolves. Crouch posed the question 'How have transposable genetic elements transformed the landscape of the *Cryphonectria parasitica* genome?' Why study transposable elements (TE)? Because TEs move about the host genome and insert into a host's DNA, they can exert a significant influence on the fitness and evolutionary potential of their host. Transposable elements are useful as genetic markers for population studies. They contribute to genetic variability of organism. And knowing what induces transposition may allow one to predict behavior of organism in given conditions.

Crypt1 is the first element that was identified in *C. parasitica* a few years ago. It is a Class II DNA transposon of the *hAT* family. It was identified as moderately repetitive probe by Milgroom in the early 1990s. It has 8-20 copies in most *C. parasitica* isolates and it is a 3.6 kb element.



It is a typical DNA transposon in that it has a single open reading frame that codes for a transposase. It is bordered on both ends by inverted repeats and direct repeats. *Cryp1* transcript in *C. parasitica* isolates with complete transposase coding sequences, suggests activity. It is active but not in all isolates of *C. parasitica*. The copy number of *Crypt1* varies based on population. In the draft sequence of the *C. parasitica* genome (EP155), 31 *Crypt1* copies of the element are present.

Crypt2 is the second element identified. It is also a Class II DNA transposon but of a different family, *fot1* family. It has more copies than *Crypt1* in most *C. parasitica* isolates. It has more defective copies than are found in *Crypt1*. It is presumed active

through presence in EST library. *Crypt2* is present in much higher copy number than *Crypt1*. Crouch found that *Crypt2* copies from *C. parasitica* are very closely related to *C. nitschkei*. There are four subgroups of this element. It appears if the elements from the two species have co-evolved.

Long terminal repeat retrotransposons (LTP). These elements are much more complex than *Crypt*. LTP have a whole compliment of genes. Instead of using DNA, they use RNA and reverse transcriptase. In terms of their structure, there are always going to be more 5'end to 3' end because of the long terminal repeats. This provides an internal clock for these elements because when they insert into the genome, the 5' LTR and 3' LTR are always going to be identical. The 5' and 3' LTR sequences provide evidence for high levels of recombination in the full length retrotransposons. Only 23% of the 39 full length retrotransposons are non-recombinant.

What was interesting about both the DNA and retrotransposons is that when they looked at their sequences, they expected to find evidence of a process known as repeat-induced point mutation or RIP. RIP is a process the genome uses—it occurs during meiosis—in which there are two copies of a single gene at any given time. They are identified by unknown mechanisms and the genome will induce transitions in the duplicated sequence. They will change C to T and G to A. They are able to recognize sequences that are as little as 80% similar to one another. Whenever there are two copies of any gene, the RIP process will pepper the sequences with C to T or G to A mutations. This will result in breaks in open reading frames. RIP is a very good way for fungi to protect against transposon proliferation. They expected to see RIP mutation in *C. parasitica* because RIP processes have been identified in many of the close relatives of *C. parasitica*. In *N. crassa,* RIP has made a significant impact in the way the organism has evolved. Not only does it target transposons in *N. crassa*, but it targets any type of gene families so it has minimized the gene families in *Neurospora*. In other fungi, RIP is not as strong a process.

Colletotrichum cereale, the fungus that Crouch mainly works with, has a canonical pattern of RIP mutation. The way to identify RIP mutation is to look for patterns of dinucleotide ratios. In RIP mutated sequences, it will be skewed relative to normal genome levels. The only skewing that occurred with *C. parasitica* was with the CGs. This suggests that, while there is no evidence for RIP, there might be methylation occurring since repeat sequences are often hotspots for methylation.

Crouch's initial question may have to be turned around to ask 'How has the genome of *Cryphonectria parasitica* transformed its transposable genetic elements?' If there was no regulation, you would expect to see a high number of transposable elements and a real increase in the size of the genome—that is not the case. There might be a number of different possibilities. First there might be *de novo* methylation of repetitive sequences—this is suggested by the under-representation of the CpGs. (CpG stands for cytosine and guanine separated by a phosphate, which links the two nucleosides together in DNA). Second, there might be dicer-mediated RNA silencing. A third possibility is post-transcriptional modification. This has recently described as a transposon silencing mechanism in *Magnaporthe oryzae*, a close relative of *C. parasitica*. Finally, there might be non-canonical RIP mutation. There is a homologue of the one gene that has been identified as participating in RIP mutation and that is the *N. crassa* rid-1(cytosine methyltransferase) gene.

Angus Dawe—New Mexico State University

Web site. Dawe has developed a website (http://chestnut.nmsu.edu/index.html) for posting information related to chestnut and NE-1015. Built into that is an email listserv system: ne1015@chestnut.nmsu.edu. This goes out to approximately 70 individuals. It can be used as a listserv and for email. Dawe will add any links to the website, within reason. He already has added Anagnostakis' chestnut lists and some pdf files.

BDM-1, an essential component of G-protein signaling in *Cryphonectria parasitica.* The reason that G-protein signaling is so important is because these are pathways that connect receptors on the cell surface to the nucleus where there can be transcriptional changes of gene expression in response to stimuli. It involved three highly conserved proteins at the 'top' end α, β, and γ. The signal trigger, activated by a cell surface receptor binds to something (yet unknown) and there is a dissociation of the βγ complex from α. These can be extremely sensitive systems.

BDM-1 is a potential regulator of G βγ signaling.

- BDM-1 is not an α, β, or γ subunit but it is something that regulates how the subunits behave
- Deletion of BDM-1 gives very similar phenotype to ΔG β
- Disruption of either bdm-1 or G β results in the reduce accumulation of G α subunit (CPG-1)
- BDM-1 is similar to other things that are also involved in G-protein signaling in other systems, not just *Cryphonectria*. Some are mammalian and they fall into a category of phosducin-like proteins which are involved in G-protein signaling pathways. BDM-1 contains clusters of amino acid identity with mammalian phosducins and phosducin-like proteins
- Conserved genomic arrangement across different fungal genera

The two genes that encode these proteins are very closely associated in the genome; they sit right next to one another. In *C. parasitica* they are about 8kb apart; there are no predictive ORFs in between them. This is maintained that way in *Neurospora, Magnoaporthe* and *Aspergillus*. There is not just a functional association between the two genes but also a physical association.

In mammalian systems the phosducin-like proteins (PhLP) are involved in either blocking or, in some cases, stabilizing the formation of the Gγ subunit. A key part of the function of this phosducin-like protein is whether or not they get phosphorylated. There is: (1) disruption of PhLP blocks G-protein signaling or mimics the disruption of the gene; and (2) phosphorylation of the PhLP by casein kinase II (CK2) plays important role in PhLP function

The tagged version of BDM-1 looks just like Ep155. BDM-1 can be dephosphorylated *in vitro* and dephosphorylated proteins are shifted in migration compared to untreated lysates. They have found that BDM-1 is a target of CK2. They are in the process of looking at mutants in *bdm-1* sequence that will alter the potential for phosphorylation. There are five possible CK2 sites. Several combinations (13) have been made so far. Why is there a concern of phosphorylation? Part of the phenotype of hypovirus infections is caused by compromised G-protein signaling pathways. From work done with Segers on microarrays, Dawe knows that the signaling pathways are

affected in the same way; the effects on one subunit also cause effects on others. BDM-1 affects CPGB-1 and in mammals, PhLP proteins are phoshorylated. Their working hypothesis is that a potential mechanism for hypovirus-mediated effects on G-proteins is through too much (or too little) phosphorylation of BDM-1. This may result in the CPGB-1 not binding properly. CPGB-1 is degraded more quickly and the γβ complex cannot form. As a result, CPG-1 is degraded more quickly and the signaling pathway is compromised. The obvious question is 'what does the BDM-1 protein look like in the presence of Ep713?' They took flag-tagged constructs and then introduced Ep713 virus by anastomosis. In either ellagic acid (a CK2 inhibitor) or in the presence of hypovirus, BDM-1 has a faster migrating form. This is also true to Euro7-infected mycelium. This raises the question as to whether there is another kinase involved. Dawe showed pictures of the phenotype of *C. parasitica* when grown on various concentrations of ellagic acid and at concentration of 1mM, the fungus stops growing. Dawe speculated that with any inhibitor, the more you add, the more things are affected, other than the target. Thus, more than just CK2 was inhibited so that at 1mM of ellagic acid, the fungus stopped growing altogether.

They are in the process of trying to get some analyses done on purified BDM-1 protein. They are using mass spec analyses at Baylor University to digest the protein into pieces. The machine will then tell them what each protein fragment weighs. Knowing the amino acid sequence, they can figure out from which peptide each fragment is derived, and hopefully, see which are phosphorylated and which ones are not. Dawe showed a 2-D gel of *C. parasitica* proteins that are phosophorylated; the 2-D gel separates not only by size, but also by charge and this gives much greater array of separation than by size alone. There are many sites in the protein that can be phosphorylated; also there are multiple kinases and multiple forms making this a complex problem. They are just now beginning to look at different arrangements of proteins from infected and uninfected strains of *C. parasitica*.

The genomic leverage will allow investigation into various areas. If kinase activity is affected by hypovirus infection, many other targets besides BDM-1 are likely affected. A whole slew of post-transcriptional mechanisms can be looked at to see changes in the way cells behave. This should be reflected in changes in the "phosophoproteome". Using 2-D gels, spots could be picked where they see the protein has been phosphorylated, the mass spec done and then a comparison to the genome sequence could be used for gene identification.

Amanda Kemp—New Mexico State University

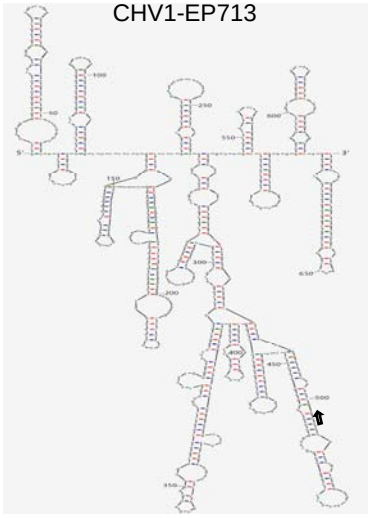
She reported on genomic regions controlling transcription of BDM-1 and CPGB-1. As has been previously published, BDM-1 and CPGB-1 are encoded by genes that are located next to one another in the *C. parasitica* genome. The gene *cpgb-1* encodes the β subunit of the G protein and *bdm-1* encodes a protein called BDM-1. Both genes are required for virulence. Knockouts of *cpgb-1* and *bdm-1* give identical phenotypes: reduced pigmentation; reduced sporulation; and, absence of G_{β} subunit. The objectives of her study were two-fold: (1) identify genomic regions that control transcription of genes; and (2) determine whether or not they have same transcriptional controls. In order to investigate whether this has any significance in relation to the control of these genes, a series of reporter constructs have been created. Each construct is based on a green

fluorescent protein (GFP) expressing vector that contains the necessary marker (hygromycin resistance) for use in *C. parasitica*. Sections of genomic DNA corresponding to regions upstream of both genes have been amplified by PCR and used to replace the gpd promoter that was built into the original vector, thus permitting expression of GPF controlled by those genomic pieces. Several constructs containing various lengths have been completed and transformations into wild-type *C. parasitica* are underway. Preliminary measurements of transcriptional activity are being performed bys using RT-PCR.

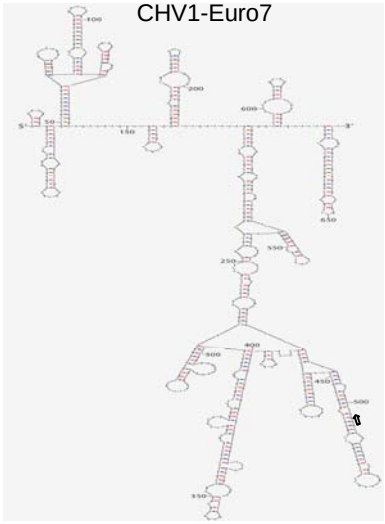
Rong Mu—New Mexico State University

She reported on the secondary structure analysis of 5' untranslated regions (UTR) of the hypovirus genome. The 5'UTR is a section of RNA before the start code; it is not translated. The 5'UTR is know to have importance for mRNA stability and translational efficiency. The potential application of hypoviruses as biological control agents is dependent on their efficacy as replicative entities with the host cell. She focused her work on CHV1-Ep713 and CHV1-Euro7. Both genomes contain two ORFs and both have similar lengths for the 5'UTR, although CHV1-Euro7 is one nucleotide shorter than CHV1-Ep713. Mu used a computer prediction and experimental support (using RNase mapping and DNA sequencing) for a putative structure model. The understanding of virus-encoded and host factors that modulate this may enable the design of more efficient and therefore more effective control agents. To this end, they have begun to examine the specific structural elements at the 5' end of the hypovirus genome that are not translated. In other viral systems, analogous regions are often important for both replication and translation. The approach has been to first predict the structure of these regions using mFold, a web accessible nucleic acid folding predictor as seen below.

Mfold prediction



ΔG=-298.61Kcal/mol
Total nucleotides: 675nt=5'UTR+180nt



ΔG=-295.43Kcal/mol
Total nucleotides: 675nt=5'UTR+181nt

Following that, extensive RNAse protection assays have been performed that permit the correlation of regions of single and double stranded nature by these empirical

measurements and the software prediction. The end result is a two-dimensional constrained diagram that likely represents a more accurate assessment of the structure. They have completed data collection and are generating constrained models for both CHV1-Ep713 and CHV1-Euro7. For future work, it will be possible to test the importance of any given structure by introducing mutations that will change it and using transfection of *C. parasitica* to assess the ability of these mutated sequences to replicate.

Michael Milgroom—Cornell University

For many years, Milgoom has been studying the population biology of *C. parasitica* motivated by the complexities of population biology as it relates to the transmission of hypviruses. Milgroom focused on three areas: (1) cloning of vegetative incompatibility genes (in conjunction with Cristina McGuire, Tom Kubisiak, Alice Churchill and Myron Smith); (2) heterokaryons and parasexuality (in conjunction with Kiril Sotivorski and Mihajlo Risteksi); and, (3) population structure in Europe (in conjunction with Kiril Sotivorski and Paolo Cortesi).

Population biology and genetics of *C. parasitica*. Vegetative incompatibility is a self-non-self recognition system in fungi that triggers cell death. It prevents heterokaryon formation and inhibits virus transmission. It is a multi-locus system with six genes identified to date. Each gene alone, if different between two strains, may cause incompatibility. There may be more loci involved; in fact there are more genes because the diversity in China and North America cannot be explained by the six genes. The reason to clone *vic* genes is to try and understand the mechanisms of vegetative incompatibility. What is their role in cell death pathways? Myron Smith's work has shown that there is an interaction between the fungus and the virus in that the virus can suppress the cell death reaction to some degree. It is possible that if we clone genes maybe we can manipulate the system and end up with better virus transmission. From an evolutionary perspective, *vic* genes or *het* genes as they are called in other fungi, for heterokaryon incompatibility—only 6-7 have been identified so far, and there are not a lot of clear patterns as to what these genes are doing.

Several years ago, Milgroom and Kubisiak published a linkage map showing five linkage groups, each showing the location of a *vic* gene that was segregating in mapping population. One of the markers they had co-segregated with *vic2*, the one they decided to clone first. They have another marker that is relatively close to *vic1* but they have not made much progress on that. The strategy was to screen a cosmid library with the marker that is co-segregating and use that marker to transform *C. parasitica*. The assay is—if you transform an isolate with the same allele at a *vic* locus you get normal transformation. But if you transform with a different allele, you cause an incompatible reaction and you get no transformation. They found a cosmid that they think is carrying the allele #2 at locus *vic2*. When they take strain Ep44 and transform it with the cosmid, they get normal transformation. When they transform a strain (EU-62) that has allele #1 with a cosmid containing allele #2, they get cell death and no transformation. They have done this with several other strains and they get the same results. They have subcloned the cosmid down to a 10kb subclone. On this subclone there are open reading frames with similarities to: (1) a putative *het* domain in *Aspergillus fumigatus;* and, (2) GTP-binding domains (as found in *het* genes *Podospora anserina).* Ongoing work in this area includes:

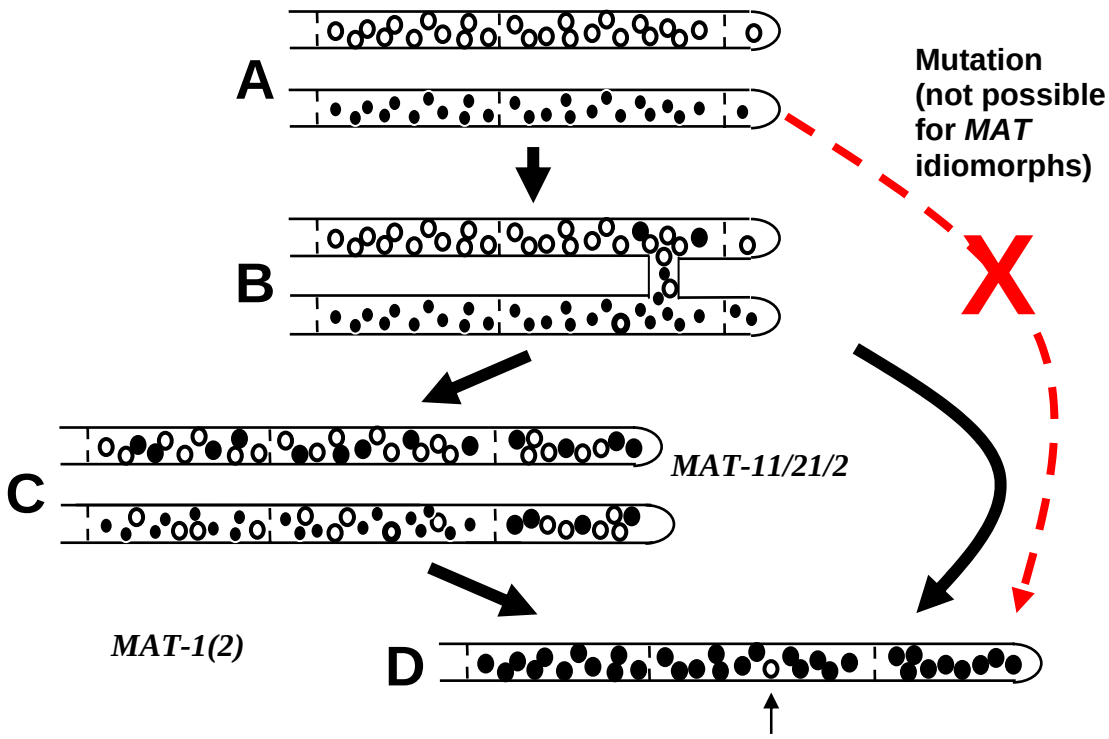
- subclone *vic2* for functional assay
- sequence cDNAs
- sequence other allele

Future work includes:

- linkage mapping to find markers for cloning other *vic* genes
  -- *C. parasitica* genome sequence
- cell death pathways
- interactions with hypoviruses
- evolution of *vic* genes

MAT heterokaryons and parasexuality. Much of the work he presented was part of Cristina McGuire's Ph.D. work. Marin Brewer has also been involved in this project. Kiril Sotirovski and Mihajlo Risteski are collaborators from Macedonia who have been working in Milgroom's lab. They stumbled on heterokaryons while looking at what they thought was selfing in *C. parasitica*. When they do a PCR assay for mating type, they can find some isolates that contain both mating type idiomorphs present in the same individuals (2 of 20 isolates examined had both idiomorphs). If they take single conidial isolates that they think are heterokaryons, they segregate into MAT-1 or MAT-2. This is a classic case of a heterokaryon. They never find a single conidial isolate with both mating types. This work was done using hyphal tip cultures to preclude mixed cultures. This is a strange finding for an Ascomycete fungus. Heterokaryons are not typical in Ascomycetes unless they are caused by a mutation. In the case of *C. parasitica*, there is no mutation involved because MAT-1 and MAT-2 alleles are very different. They are so different that they are not called alleles but idiomorphs.

How do *MAT* heterokaryons form**?**



In the above diagram, the white and black circles represent different MAT idiomorphs. When fungi come in contact with one another (panel B), they can form anastomosis bridges. Mixing can occur (panel C). They sometime find events, like panel D, in which the results are really skewed; they have barely detectable signals from one idiomorph. Heterokaryones do not form by mutation because the two idiomorphs are almost completely dissimilar in sequence. Stable heterokaryons do not form between vc types because of the programmed cell death. The overlying question is do heterokaryons form between vc types in *C. parasitica*. Heterokaryon formation does not occur in the laboratory. Dennis Huber in Fulbright's lab could not get heterokaryon formation in the lab and Myron Smith, using different vic genes, also got no formation. One gene actually allows heterokaryon formation, but five of the six genes do not. However, when a clonal population of *C. parasitica* from West Salem, WI was analyzed, they found evidence for heterokaryon formation. They believe this occurred between vc types. Milgroom has tried to confirm this finding by looking at other clonal populations in Macedonia and Greece. He and Sotivorski looked at about 800 isolates from Macedonia and Greece and found that 94% were in same vc type (EU-12) and 97% were *MAT-1*. Is this a giant clonal population and are there any heterokaryons? They used 11 different SCAR markers and found the following:

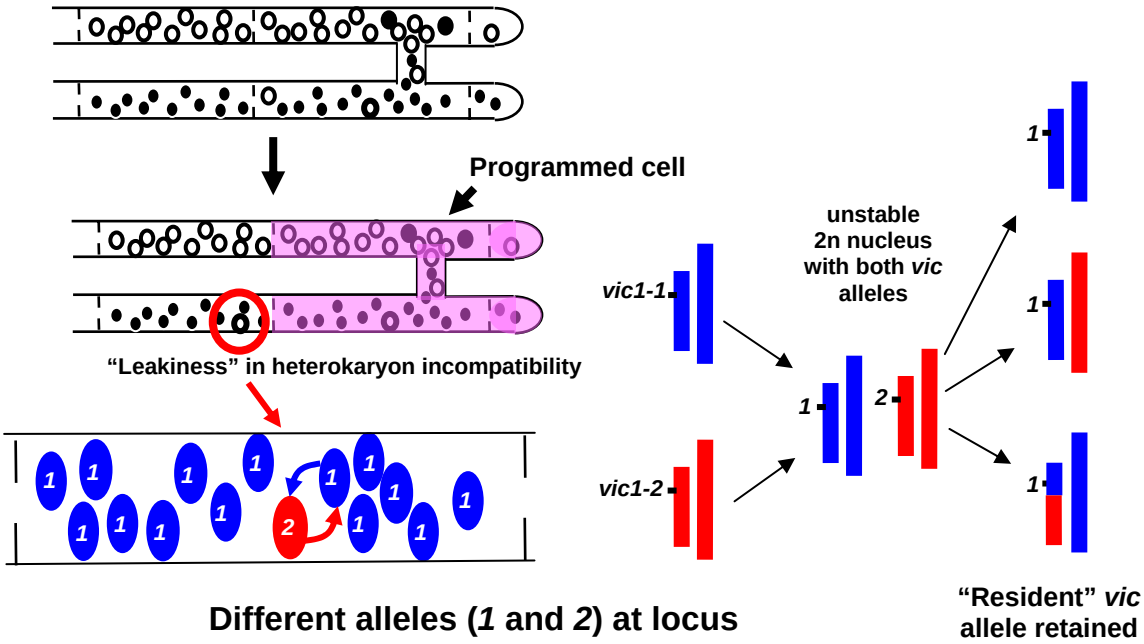
Sample Location	**Number of Samples**	**EU-12**	**Six other vic types**
Greece	53	53	0

Macedonia	134	102	32
Bulgaria	27	25	2
Romania	27	26	1
Sicily	114	91	23
Turkey	20	0	20

The above sites were chosen because they knew that most of the fungal population was EU-12; the exception was Turkey and its main population is EU-1. From the 375 isolates they genotyped, at 11 SCAR loci and 6 *vic* loci, they found only 9 haplotypes. The same haplotyes were found all across Europe. The EU-12 isolates from Macedonia had the same SCAR loci as isolates in Bulgaria and Sicily, etc. That was also true for *vic* loci. No evidence was found for recombination between vc types, even though perithecia were found in some locations. Of the 375 isolates, only three were heterokaryons, one from Macedonia and two from Italy.

In the one Macedonian population where they found the heterokaryon, there are two clones, EU-12 and EU-2. They looked at hyphal tip isolates of a heterokaryon and found it had both alleles at all six loci. When they took single conidial isolates from them, they ended up with three different haplotypes, none of which were identical to the EU-12 or EU-2 clones. It looks like the EU-12 and EU-2 clones somehow fused to form a heterokaryon and the single conidial isolates are recombinants. This is very reminiscent of parasexuality. The problems how do you get stable heterkarons formed between different vc types. It is not supposed to happen. He does not have data, but he has a hypothesis. In parasexuality, you have to get heterokaryons formed so that you have two unlike nuclei in the same thallus. Then the two unlike nuclei fuse to form a diploid. In a normal sexual cycle, there is meiosis, but with parasexuality, there is a random loss of chromosomes by mitotic nondisjunction. There is some recombination that can occur by mitotic crossing-over. Recombination then occurs during the haploidization process. In terms of recombination, you get independent assortment of chromosomes, as you might in meiosis. His diagram of the parasexual model is below:

Model: parasexuality between incompatible



Different alleles (*1* and *2*) at locus

As shown above, getting two unlike nuclei in the same thallus is difficult to explain because of the programmed cell death. Thus, there might be some leakiness involved. The alternative hypothesis is that heterokaryons form during normal mating—somehow mating is aborted before meiosis. Or somehow the dikaryon condition persists after mating, but this would only occur between MAT-1 and MAT-2 individuals. Somehow between plasmogamy and heterokaryon formation, the vegetative incompatibility reaction is shut down. There may be some sort of suppressor of the incompatible reaction, but he would expect is a different type of incompatibility reaction. To date, there is no evidence for a suppressor. Also, the presence of viruses could be suppressing the vic reaction. Myron Smith has shown that viruses do not suppress the reaction entirely, but they delay it. Milgroom predicts that a diploid nucleus with both alleles at an incompatibility locus, one of the alleles is lost preferentially to try and maintain the same *vic* allele as in the resident. There would be selection against the nuclei with the wrong allele. Future work includes testing the parasexual hypothesis under lab conditions. He predicts a loss of paternal markers with paternal *vic* allele.

Laura Georgi—Clemson University

Chromosome walking towards genes for resistance to blight. Chromosome walking is a technique to explore the chromosomal sequences that are contiguous to a know segment of DNA. She is collaborating with Carlson's group at Penn State to clone the genes for blight resistance from Chinese chestnut. They have been looking at a couple of QTLs on linkage groups B and F. Georgi constructed a BAC library which is an ordered collection of random fragments of chestnut genomic DNA propagated in *E. coli*. She is using RFLP markers CD175 and CD145. Robert Bernatzky's sequence for marker CD175 resembles a gene for Phosphoglucan, water dikinase (involved in starch metabolism) from *Arabidopsis thaliana*. It's the right size, and the internal EcoRV site is

also consistent with the original marker. BAC direct sequencing uncovered a Bcl1 site in *C. mollissima* 'Mahogany' sequence not present in the *C. dentata* cDNA. (The base pair difference responsible is not silent.). Kelly Deitrick's (Penn State) genotypes for this CAPS marker (cleaved **a**mplified polymorphic sequences—which can be used for genetic mapping, in this case an *Arabidopsis* gene to one of the chromosome arms) matched up beautifully with the original CD175 RFLP. For CD145, Carlson's group sequenced a collection of plasmids, including CD145, used for RFLP probes on the original interspecific map. Although they obtained the same sequence for CD145 and for CD146 (unmapped), the size and predicted EcoRI fragments are consistent with the sequence being CD145. At last year's meeting Don Nuss informed Georgi about another collection of the RFLP clones. She obtained them from Timothy McKechnie. There is a sequence for CD146 in that group but it does not match the 146 from Carlson's group. The PSU CD145 sequence looks like a subunit (D chain) for a F0ATPase.

CD145 is a little mRNA (507 bp from AUG start to UGA stop), but the gene has four introns. The intronic sequences differ quite a bit between *C. dentata* Long Branch and *C.mollissima* Mahogany. The second intron in *C. dentata* (Long Branch and Brasstown Bald) has four Bfa1 sites; *C. mollissima* Mahogany and Nanking have three. Unfortunately, CAPS didn't work in the mapping population. CD175 hybridized to seven *C. mollissima* BACs, while CD145 hybridized to thirteen.

Georgi is looking at the Poplar genome sequence (which is completely sequenced); there is evidence that woody plants may be evolving more slowly on a chronological scale than herbaceous plants. Georgi cited G.A. Tuskan and other's workers [Science 313, 1596 -1604 (2006)], who completed the draft genome of the black cottonwood tree, *Populus trichocarpa*. Integration of shotgun sequence assembly with genetic mapping enabled chromosome-scale reconstruction of the genome. More than 45,000 putative protein-coding genes were identified. Analysis of the assembled genome revealed a whole-genome duplication event; about 8000 pairs of duplicated genes from that event survived in the *Populus* genome. A second, older duplication event is indistinguishably coincident with the divergence of the *Populus* and *Arabidopsis* lineages. Fossil evidence for this is that Poplars are around 60 million years old. However, if only the sequences are examined, the molecular clock has been ticking for only 8-12 million years.

Georgi showed a cartoon of Poplar chromosomes VIII and X and she said that there is a sequence match between the chromosomes. The main organism of interest in the Abbott lab is not chestnut but peach. They are in the constructing a physical map of peach. The link is that peach, a member of Rosales, is much closer to the *Fagales* than Malpighiales (*Populus*). This may be a better scaffold in term of cloning genes. Some peach BACs hybridized with cDNA probes, CD145 and CD175. Georgi's BAC end sequences uncovered a number of genes in both Chinese chestnut and peach that show conservation of synteny with *Populus*. That's where genes that are near each other on a chromosome in one species are also near each other in another. Her motivation in looking at these related species is to find the genomic equivalent of a grown-up leg to grab onto, in hopes she can stand up and take a step or two. Georgi's plans include sequence one of the CD175-positive BACs and identify more markers linked to the resistance loci and continue the chromosome walking.

Fenny Dane, Department of Horticulture, Auburn University (submitted report)

Phylogenetic analysis using sequences of six chloroplast DNA regions of American chestnut (*C. dentata*) and Allegheny and Ozark chinkapin (*C. pumila* var. *pumila,* and var. *ozarkensis*) populations across the species range was conducted to study the origin and historical processes responsible for the distribution of genetic diversity in contemporary populations. Levels of intraspecific variability are high in Allegheny chinkapin populations in Virginia and Georgia, and American chestnut populations only in southern regions. Two main lineages are apparent within American chestnut populations, which are closer related to Allegheny chinkapin haplotypes from the southern Appalachian region than to each other. Mutational events must have long preceded the effects of the Last Glacial Maximum. Levels of diversity were lower in the Ozark chinkapins and Florida panhandle for Allegheny chinkapins. Intermediate Allegheny chinkapin haplotypes were detected in one Snakeden Mountain population in Virginia pointing to the southern Appalachian mountain range as potential refugium and suture zone of two different chinkapin lineages. Hybridization and introgression in southern refugia leading to extensive sharing of the chloroplast genomes between *C. pumila* var. *pumila* and *C. dentata* must have occurred over time.

C. dentata expressed sequences deposited in the GenBank were searched for redundancy and used for the development of species specific nuclear markers. Five ESTs were selected for in depth sequencing analysis. Intraspecific variability was detected at a few regions, while heterozygosity at other regions complicated their use. One informative SNP (transversion) was detected at region 17, which can be used as a species specific marker to distinguish *C. dentata* from *C. pumila*.

Bob Nowierski-USDA-CSREES

Invasive species, funding opportunities, and regulatory reform. Nowierski thought that members of the group had a little better idea of the programs at the national level—that if members want to engage in invasive species activities or if there were any suggestions or concerns about how to improve requests for applications across the different grant programs, please let him know.

With regard to invasive species, nearly every terrestrial, wetland, and aquatic ecosystem in the U.S. has been invaded by non-indigenous species. Economic losses are estimated at $137 billion/year. Invasive species constitute one of the most s serious economic, social, and environmental threats of the 21st Century.

He showed pictures of some invasives: European ash borer, Asian long-horned beetle, chestnut blight, sudden oak death, soybean rust, old world climbing fern, kudzu, purple loosestrife, spotted knapweed, diffuse starthistle, yellow starthistle, purple starthistle, gorse, giant hogweed, giant salvinia, water hyacinth, alligator weed, and Eurasian watermilfoil. Nowierski encouraged adoption and implementation of IPM including:

- Utilize all available PM tools in the most cost-effective and environmentally sound fashion possible
- All pest management strategies have potential risks
- Need to weigh benefits of a management strategy with its potential risks
- Risks of doing nothing

Coordination of invasive species efforts at the national level includes inter-agency cooperation, federal coordinated advisory committees and cooperation with colleagues.

In terms of program structure, President Clinton signed an executive order in 1999 creating the National Invasive Species Council (NISC) which is chaired by the Secretaries of Agriculture, Commerce and Interior. It is co-chaired by the Secretaries of: State, Treasury, DOD, DOT, Administrators for EPA, Administrators for USAID, Dept. Homeland Security and NASA. This order also authorized the formation of the Invasive Species Advisory Committee. Each of the three co-chairs has liaisons. The USDA Senior Invasive Species Coordinator is Hilda Diaz-Soltero and she provides a liaison rote between the Department of Agriculture and NISC. Diza-Soltero coordinates invasive species efforts for the following USDA agencies: APHIS, ARS, CSREES, ERS, FS, NRCS. The invasive species website is: http://www.Invasivespeciesinfo.gov.

For inter-agency cooperation, there are the national invasive species (I.S) management plan subcommittees and the budget crosscut for invasive species, FT08. They identify the most important IS issues and activities are shared across federal agencies, invasive species efforts are prioritized and they develop and assess performance measures against IS.

National Invasive Species Management Plan – '01 (57 action items; being reprioritized) Subcommittees:

- Leadership and Coordination
- Prevention
- Early Detection and Rapid Response
- Control and Management
- Restoration
- International Cooperation
- Research
- Information Management
- Education and Public Awareness

Invasive Species Budget Crosscut

- Brown tree snake
- Saltcedar
- Emerald ash borer
- Leafy spurge / yellow starthistle
- Sudden oak death
- Asian carp
- Ballast water
- Prevention through education
- Aquatic area monitoring strategies
- Early detection and rapid response
- Innovative control methodologies

CSREES is also involved with other PM activities such as:

- National Invasive Species Management Plan
- Early Detection and Rapid Response Subcommittee (Bewick – Co-Chair)
- Communications andOutreach Subcommittee (Bewick – Co-Chair)
- Control and Management Subcommittee (Nowierski – Co-Chair)
- Smoothing the Regulatory Process Subcommittee (Delfosse – ARS, and Nowierski Co-Chairs)

- TAG (Technical Advisory Group) for the biological control of weeds (Nowierski, Bewick)
- APHIS-PPQ permitting board of advisors (Nowierski)

There are opportunities for pest management and greater research communities to engage in invasive species issues. There are activities to prevent agriculture bioterrorism; management of invasive species through participation in the National IS management plan subcommittees; and, there are funding opportunities.

The National Plant Diagnostic Network was developed after 911. The idea is to have different regional hubs that are in turn connected to diagnostic laboratories. The idea is to have a quick response. If first responders detect something unusual, they can take it to the diagnostic center in their region. Beltsville, MD is the backup for identification. This system was used for the Pink hibiscus mealybug (PHM):

- 900,000 hibiscus plants were shipped to retail stores in 36 states in '04 (PHM later found in KS, LA, NC)
- PHM attacks over 300 host plants (vegetables, fruit trees, citrus, ornamentals)
- Effective parasitoids (mass-rearing: CA, FL, Puerto Rico)
- Critical Issues funding - $50,000
- Steering Committee, diagnostics training workshop, teleconferencing training, pest alert factsheets

Funding Opportunities - CSREES:

NRI (National Research Initiative):

- Agricultural Res. Enhancement Awards
    - Postdoctoral Fellowships
    - New Investigator Awards
    - Strengthening Awards
- Arthropods & Nematodes: Organismal Biology (1-17-07)—focus on research
- Microbial Biol. : Pl.- Microbe Assoc. (12-14-06))—focus on research
- Weedy, Invasive Species (letter of intent: 12-06/06; proposals due: 2-14-07))—focus on research and integration
- Arthropods & Nematodes: Suborganismal Biology and Tools (6/05/07))—focus on research
- Plant Biosecurity (6/05/07)—focus on integration

**22% Integrated Component (Res., Ed., and Extension) available for a number of NRI Programs ("IFAFS-like $$)

Pest Management Programs:

- CAR – Crops at Risk
- CENTERS – Regional IPM Centers
- OTP – Organic Transitions Program
- MBT – Methyl Bromide Transitions
- RAMP – Risk Avoidance and Mitigation Program
- Critical Issues – Critical and Emerging Pests and Diseases (Plants)
- IR- 4 – Minor Crop Pest Management Program
- PMAP – Pest Management Alternatives Program
- RIPM – Regional Integrated Pest Management Program
- SARE – Sustainable Ag. Res. and Education Program

The associated website is: http://www.csrees.usda.gov/fo/funding.cfm

Regulatory reform—recommendations-internal management review of APHIS-PPQ permitting process includes:

- Rescission of prohibition on hand-carrying and bonded carrier requirement
- Establish a permitting board of advisors
- Improve customer service
- Regulatory reform

PPQ permitting board of advisors:

- APS – Bob Martin, USDA/ARS, Corvallis, OR
- DHS/CBP – Mike Oraz, Washington, DC
- ESA – Alma Solis, USDA/ARS, Beltsville, MD
- ISAC – Hilda Diaz-Soltero, USDA Senior Inv. Spp. Coord., Wash., DC
- NPB – Matt Travis, Annapolis, MD
- NPB – Kimberly Merenz, Helena, MT
- PPQ/ER – A. Wildman, PPQ/Florida
- PPQ/WR – Stacy Scott, Fort Collins, CO
- USDA/ARS – Kay Simmons, Beltsville, MD
- USDA/CSREES – Robert Nowierski, Washington, DC
- USFW – John Fay, Endangered Spp. Unit, Arlington, VA
- PPQ Biotech. Regulatory Service - ?
- EPA - ?
- APHIS-PPQ – Alan Green, Riverdale, MD (non-voting)
- APHIS-PPQ – Mike Firko, Riverdale, MD (non-voting)
- APHIS-PPQ – Bob Flanders, Riverdale, MD (non-voting)

Top issues include:

- Regulatory reform
- Written guidance on permitting procedures
- Commercial carrier issues – refusal to ship
- Containment facility issues (operation/ training)
- Improved communications on regulatory changes
- Live plant pest exhibits / Insect zoos
- Soil permitting and nematode issues
- Tightening regulations on umbrella permits (e.g., for commercial companies with Regulatory Officers who may reside in different states)

Regulatory changes for APHIS-PPQ in 2007 include:

- e-permits
- Changes to shipping label—now reads "living regulated organisms' rather than 'live plant pests and pathogens'
- Will eventually have a barcoding system on shipment label (for CBP and APHIs-PPQ inspection stations
- Regulatory change working group—changes to Plant Protection Act of 2000—biocontrol language; risk-based system for regulatory oversight

Business Meeting

Brad Hillman was elected Chair-elect. Dennis Fulbright will chair the 2008 meeting, and agreed to host the meeting at Frankfort, MI in mid-October (possibly 16-19th). Fulbright does not want to conflict with the meeting in Beijing, China, scheduled for Sept. 15-18, 2008. There was discussion about renewing the project. The following members agreed to act as a steering committee: Bill MacDonald, Sandra Anagnostakis, Dennis Fulbright, Fred Hebard, Hill Craddock, Paul Sisco, and Don Nuss. Michael Gold was suggested as someone who might be willing to join the steering committee. MacDonald agreed to provide deadlines for the vision statement and coordinate the writing of the 2009-2013 project proposal.

A tour of three of The American Chestnut Foundation's Meadowview farms, led by Fred Hebard and Bob Paris, was conducted following the meeting. Following the farm tour, dinner followed at the historic Abingdon Tavern followed by an optional evening at the Barter Theater to see the production, 'Harry Chapin: Remember the Music'.

Respectfully submitted,
Mark Double, October 2007

Milestone Accomplishments

2004 Milestones Accomplished:

- Nutritional analyses of nuts was conducted from orchard selections and cultivars of chestnut.(Anagnostakis)
- Hypovirulent strains of *C. parasitica* were developed and deployed for blight control on native chestnut trees at each of three clear-cut forest areas and one nursery are planted with hybrid chestnut trees (Anagnostakis)
- Site was selected for release of Euro 7 transgenic strains in West Virginia (MacDonald and Nuss)
- Breeding program begun for selecting improved protein content, peelability, and blight resistance in high yielding orchard-based chestnut cultivars (Fulbright—Endowed chestnut experiment station, Rogers Reserve Farm, MI).
- Nuts collected for nutritional analyses and size comparison from orchard selections and cultivars of chestnut (Anagnostakis).
- Hypovirulent strains of *C. parasitica* developed and deployed for blight control on native chestnut trees at each of three clear-cut forest areas and one nursery area planted with hybrid chestnut trees (Anagnostakis).
- Scion wood collected from WI American chestnut trees surviving well with hypovirulence in the *C. parasitica* population, grafted onto root stocks in MI (Fulbright and Jarosz).
- Site selected for release of new transgenic-hypovirulent strains of *C. parasitica* in WV (MacDonald and Nuss).

2005 Milestones Accomplished:

- Market research analyses were completed and the findings reported (MO, MI). New selections of experimental lines from MO, CT, TN and MI are being established. (Gold)

- Orchard of advanced backcross chestnut for assessment of host resistance with hypovirulence was established in WV, albeit the planting failed due to raccoon predation of nuts (MacDonald and Hebard)
- Characterization of the role of hypovirus p29 in virus RNA accumulation in *C. parasitica* and virus transmission through conidia of the fungus (Nuss)
- Generation of polyclonal antibodies against 5 overlapping regions of hypovirus ORF B and construction of *a C. parasitica* database (Nuss)
- Evaluation and comparison of improved methods of hypovirus introduction into cankers under field conditions. Sequencing and genetic structure analyses of a gene cluster for anthraquinone pigment biosynthesis in *C. parasitica* completed, and targeted gene knockout strains constructed for the PKS gene and other genes to determine gene function, and studies conducted of pigment gene knockout mutants for possible phenotypic changes (Churchill).
- More American chestnut scions collected in WI (as in 2004) and grafted on trees in MI (Fulbright and Jarosz).
- Orchard established in WV with advanced, back-cross chestnut trees from VA for assessment of host resistance with hypovirulence in the *C. parasitica* population (MacDonald and Hebard).
- Cryparin, the type II hydrophobin of *Cryphonectria parasitica* were shown to be essential for stromal pustule eruption through the bark of the host, *Castanea dentate* (VanAlfen).

2006 Milestones Accomplished:

- Publication of a *C. parasitica* EST database containing approximately 2500 ESTs (Nuss)
- Demonstration that hypovirus p29 suppresses RNA silencing in *C. parasitica* and in heterologous plant system, the first report of a mycovirus-encoded suppressor of RNA silencing (Nuss—not proposed)
- A proposal to sequence the *C. parasitica* genome was approved by the Department of Enegy Community Sequencing Program. A draft of the *C. parasitica* genome sequence should be available to the research community in 2007 (Nuss, Churchill, Milgroom).
- A proposal to develop more genetic markers and a better genetic map for the Fagaceae was approved by the National Science Foundation's Plant Genome Research Program. The results of the four-year project will be posted at (Carlson, Sisco, Hebard, Anagnostakis)
- Chestnut market analyses completed and findings reported (Gold)
- New processed chestnut products introduced (Fulbright, Gold)
- New chestnut cultivars established in several cooperating locations (Fulbright, Gold)
- First transgenic American chestnut was outplanted (Powell).
- Role of canker age and vegetative compatibility on the perpetuation of hypoviruses determined, following their introduction into forest chestnut trees (MacDonald).
- Publication of a *C. parasitica* EST database containing approximately 2500 ESTs (Nuss and Dawe).

- Cloned Cpkk1 and Cpkk2, mitogen activated protein kinase kinases from *C. parasitica.* Raised polyclonal antibodies to Cpkk1 and Cpkk2. Cpkk1 found to be expressed under conditions of active mycelial growth. Cpkk1 was more abundant in the CHV1 infected strains, and persisted longer in the growth cycle of the fungus than it did in unifected strains (VanAlfen).
- CHV1 protease p29 found to associate with vesicle membranes of *C. parastica* and behaves as an integral membrane protein of the vesicular fraction derived from the fungal trans-Golgi network (TGN). The viral dsRNA, helicase, polymerase and p29 copurify with the TGN, suggesting that the virus utilizes the fungal TGN for replication. Fractionation analysis of p29 deletion variants showed that sequences in the C terminal of the protein mediate membrane association. The C-terminal portion of the protein (Met-135-Gly-248) is sufficient for membrane association and is enough to direct p29 to the TGN vesicles in the absence of other viral elements (VanAlfen).

2007 Milestones Accomplished:

- Demonstrated that RNA silencing serves as an antiviral defense mechanism in *C. parasitica* (first example for any fungus) against hypoviruses and mycoreoviruses (Nuss).
- 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 2007 (MacDonald and Hebard).
- Study to assess the saprophytic activity of *Cryphonectria parasitica* was initiated (MacDonald).
- Study using two chinquapin species to look at resistance to chestnut blight and gall wasp resistance was initiated (Anagnostakis and Schlarbaum).
- Treatments to produce high quality somatic embryos of American chestnut were determined (Merkle).
- Study to estimate the number of American chestnuts in Ontario Canada was completed (Boland, Dale).
- A breeding program, with the goal of having American chestnuts that are 75% Canadian origin in 20 years, was established (Dale).
- A new chestnut cultivar, 'Labor Day', was developed for the cold midwest climate (Fulbright).
- The father tree program was instituted utilizing pollen from chestnut in southern states (Craddock, Hebard and Paris).
- Corn, tobacco and grass plots were established to assess the success of American chestnut when outplanted in areas other than forests (Paris).
- Open pollinated progeny tests were established to reveal requirement necessary for reestablishment of American chestnut (Schlarbaum).
- A tool for the rapid assessment of foliar nitrogen and for the diagnosis of nitrogen deficiency of 'Qing' Chinese chestnut trees was identified (Gold, Warmund and Hunt).
- 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 (Nuss).

- Pigment knockout mutants of *C. parasitica* used to assess role(s) of pigments in fitness and competitive abilities in chestnut, and gene knockout strains for other PKS genes cloned (Churchill).
- Hypoviruses found in native *C. parasitica* strains analyzed for evidence for spontaneous infection or long-distance transmission based on molecular sequence.
- MI grafted (5-year-old) American chestnut selections from WI inoculated with *C. parasitica* to determine reaction to various strains (Fulbright and Jarosz).
- Experiments initiated to assess roles of other *C. parasitica* PKS genes in virulence, fungal development, and chemical defense (VanAlfen).
- Naturally occurring chestnut bark microorganisms analyzed for their antagonism toward *C. parasitica*.
- Silencing of *Kex2* found to significantly diminish the virulence of *C. parasitica* (VanAlfen).
- A dwarf chestnut tree with unknown germplasm (Asian) was identified and grafted. It is male sterile, produces dwarf offspring but at an unknown ratio to normal offspring (Fulbright).
- Hypovirulent strains were isolated from non-lethal cankers found at the Crystal Lake (Benzie County) site that treated with the GH2 hypovirulent strain in the 1980's (Fulbright).

List of Publications 2006-2007

Cernusca, M.M., M.A. Gold and L.D. Godsey. 200x. Influencing consumer awareness through the Missouri chestnut roast. Journal of Extension (submitted April 2007).

Churchill, A.C.L., Milgroom, M.G., and D.L. Nuss. 2007. Genomic sequencing of the chestnut blight fungus *Cryphonectria parasitica*. Joint Genome Institute User's Group Meeting, Walnut Creek, CA, March 28-30, 2007 (abstract).

Cooper, W.R. and L.K. Rieske. 2007. Community associates of an exotic gallmaker, *Dryocosmus kuriphilus*, (Hymenoptera: Cynipidae) in eastern North America. Annals of the Entomological Society of America 100. 236-244.

Deng, Fuyou, T.D. Allen and D.L. Nuss. 2007. Ste12 transcription factor homologue CpST12 is down-regulated by hypovirus infection and required for virulence and female fertility of the chestnut blight fungus, *Cryphonectria parasitica*. Eukaryotic Cell 6:235-244.

Deng, Fuyou, T.D. Allen, B.I. Hillman and D.L. Nuss. 2007. Comparative analysis of alterations in host phenotype and transcript accumulation following hypovirus and mycoreovirus infections of the chestnut blight fungus, *Cryphonectria parasitica.* Eukaryotic Cell 6: 1286-1298.

Gold, M.A. and R. McCoy (eds). 2007. The Chestnut Grower. Newsletter of the Chestnut Growers of America. Vol. 9, No. 1-2, Winter, Spring 2007.

Gold, M.A. and M. Hall (eds). 2007. The Chestnut Grower. Newsletter of the Chestnut Growers of America. Vol. 9, No. 3-4, Summer, Fall 2007.

Hunt, K. L., M. A. Gold, and M. R. Warmund. 2007. Chinese chestnut cultivar performance in Missouri. Annual. Rpt. Northern Nut Growers Assn. (in press).

Haiyan Lin, Xiuwan Lan, Hong Liao, T.B. Parsley, D.L.Nuss, and Baoshan Chen. 2007. Genome Sequence, Full-Length Infectious cDNA Clone, and Mapping of Viral Double-Stranded RNA Accumulation Determinant of Hypovirus CHV1-EP721. Journal of Virology 81:1813-1820.

Jeffers, S.N., J.B. James and P.H. Sisco. 2007. Screening for resistance to *Phytophthora cinnamomi* in hybrid seedlings of American chestnut. 4th IUFRO Phytophthoras in Forest and Natural Ecosystems, Asilomar, CA. http://nature.berkeley.edu/IUFRO2007/phytophthora/abstracts/32_jeffers.pdf

Keesey I.W. and Bruce A. Barrett. 2006. "Preliminary data on the seasonal occurrence and oviposition preferences of the lesser chestnut weevil, *Curculio sayi*, in mid-Missouri." Entomological Society of America, Annual Meeting; Indianapolis, IN; 11 December 2006. Oral presentation.

Kubisiak, T.L., C. Dutech and M. Milgroom. 2007. Fifty-three polymorphic microsatellite loci in the chestnut blight fungus, *Cryphonectria parasitica*. Mol. Ecol. Notes 7:428-432.

Lang, P., F. Dane, T.L. Kubisiak, and H.W. Huang. 2007. Molecular evidence for an Asian origin and a unique westward migration of species in the genus *Castanea* via Europe to North America. Molecular Phylogenetics and Evolution 43: 49-59.

Liu, Y.-C. and M.G. Milgroom. 2007. Diversity of viruses in *Cryphonectria parasitica* and *C. nitschkei* in Japan and China, and partial characterization of a new chrysovirus species. Mycological Research 111:433-442.

Liu, Y.-C. and M.G. Milgroom. 2007. High diversity of vegetative compatibility types in *Cryphonectria parasitica* in Japan and China. Mycologia. 99:279-284.

Merkle, S.A., G.M. Andrade, C.J. Nairn, W.A. Powell and C.A. Maynard. 2007. Restoration of threatened species: A noble cause for transgenic trees. Tree Genetics and Genomes 3: 111-118.

Papazova-Anakieva, I., K. Sotirovski, P. Cortesi and M.G. Milgroom. 2007. Horizontal transmission of hypoviruses between vegetative compatibility types of *Cryphonectria parasitica* in Macedonia. Eur. J. Plant Pathology (in press).

Rieske, L.K. 2007. Success of an exotic gallmaker, *Dryocosmus kuriphilus*, on chestnut in the USA: a historical account. EPPO Bulletin 37, 172-174.

Rothrock, R.E., L.D. Polin-Mcguigan, A.E. Newhouse, W.A. Powell, W.A. and C.A. Maynard. 2007. Plate flooding as an alternative *Agrobacterium*-mediated transformation method for American chestnut somatic embryos. Plant Cell, Tissue and Organ Culture 88: 93-99

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

Sudo, K., McLane, H.L., Krasnoff, S.B., Anagnostakis, S.L., Gibson, D.M., and A.C.L. Churchill. 2007. Characterization of a polyketidesynthase gene of the chestnut blight fungus by *Agrobacterium tumefaciens*-mediated targeted gene disruption. American Phytopathological Society Annual Meeting, San Diego, CA, July 28-August 1, 2007 (abstract) [http://www.apsnet.org/meetings/2007/abstracts/a07ma686.htm].

Sudo, K., McLane, H.L., Krasnoff, S.B., Anagnostakis, S.L., Gibson, D.M., and A.C.L. Churchill. 2007. *Agrobacterium tumefaciens*-mediated disruption of a polyketide synthase gene in *Cryphonectria parasitica*. Fung. Genet. Newsl. 54, #260 (abstract). http://www.fgsc.net/asil2007/xxivFGCposterAbs.htm], XXIV Fungal Genetics Conference, Pacific Grove, CA, March 20-25, 2007.

Welch, A.J., A.J. Stipanovic, C.A. Maynard, and W.A. Powell. 2007. The effects of oxalic acid on transgenic *Castanea dentata* callus tissue expressing oxalate oxidase. Plant Science 172:488-496.