NE-1015/NE-1033 Technical Committee Meeting
Biological Improvement, Habitat Restoration, and Horticultural Development of Chestnut by Management of Populations, Pathogens and Pests

Chimney Corners Resort, Frankfort, MI
October 18-19, 2008

Attendance:

California:	Debora Jacob-Wilk, Pam Kazmierczak (UC Davis)
Connecticut:	Sandra Anagnostakis (Connecticut Agricultural Experiment Station)
Maryland:	Gil Choi, Donald Nuss (University of Maryland Biotechnology Institute, Shady Grove)
Michigan:	Dennis Fulbright—Chair, Susan Howard, Andrew Jarosz, Carmen Medina-Ferrer, Josh Springer (Michigan State University)
Mississippi:	Tom Kubisiak (USDA-FS)
Missouri:	Michele Warmund (University of Missouri-Columbia)
New Jersey:	Bradley Hillman—Chair-elect, Jo Anne Crouch, Nrupali Patel (Rutgers University)
New Mexico:	Angus Dawe, Rachel Acuña, Hilary Boyer, Gloricelys Rivera, (New Mexico State University)
New York:	Alice Churchill, Michael Milgroom (Cornell University), Steven Jakobi (Alfred State College)
North Carolina:	Paul Sisco (TACF, Asheville)
Pennsylvania:	John Carlson (Penn State University), Mike Marshall (Shippensburg University)
Tennessee:	Leila Pinchot (University of Tennessee)
Virginia:	Fred Hebard (TACF, Meadowview)
West Virginia:	William MacDonald, Mark Double (West Virginia University), Bob Paris (TACF-Beckley)

The meeting was called to order by Chairman Fulbright at 9:00 am on October 17, 2008 at Chimney Corners Resort in Frankfort, MI. Fulbright stated that Chimney Corners was the site of the first NE-140 regional project meeting in 1982. Jim Rogers, Jr., owner of the Chimney Corners Resort, welcomed the group to Frankfort. Mr. Rogers told the group that chestnut trees on the resort's property were planted in 1910 by his grandfather. Chestnut blight killed most of the stems by the 1980s. Fulbright introduced hypovirulent isolates, mostly Grand Haven 2 from 1981-1988 and the regional project attendees were able to see signs of recovery in the stand. Fulbright stated that the NE-1015 technical committee officially was reclassified as NE-1033 on October 1, 2008. Fulbright acknowledged the fact that the NE-1015 technical committee was honored with the 2008 *Award for Excellence in Multistate Research*, given by the Northeastern Regional Association of State Agricultural Experiment Station Directors. Acknowledgement was given to Michael Vayda, the project's administrative advisor, for submitting the project for the award.

<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.

Dennis Fulbright—Michigan State University

Microbial decay of fresh chestnuts and its management in Michigan. Fulbright is trying to find chestnuts that have a long shelf life. Two types of mold have been noticed—external mold on the shell and internal decay. Nuts can experience decay before they come off the tree. Surface mold, which may have nothing to do with internal mold, may cause the nuts to be unacceptable to a produce manager. A mantle of white mycelium can be washed of the exterior of nuts very easily and the nut inside may be fine; however, they are not marketable. Fulbright showed slides of internal kernel decay, typical of *Penicillium*. Nut rot in Europe or Australia is caused by *Phomopsis* (black rot of chestnut). This organism is not a problem in the U.S. The problem in the U.S. is several fungi; they either as the cause of the rot, or they move in quickly after the collapse of the tissue. A survey of organisms was conducted and there are many fungi, yeast and bacteria that have been isolated from chestnuts. Fungal rotting is the most serious post-harvest problems in chestnuts around the world. Some of the fungi isolated are: *Penicillium* spp.; *Botryris cinerea; Gibberella zeae; Phomopsis castanet; Fusarium* spp.; and, *Ciboria batschiana* (=*Sclerotinia pseudotuberosa*). *Fusarium, Gibberella* and *Penicillium* can cause mycotoxin problems. The internal rot problems they have witnessed are mostly in European/Japanese hybrids; however, the fungi also can be found in Chinese chestnuts. Fungal counts peaked at 90 days of storage (at 4C) on nuts that never touched the ground. They found that touching the ground increases fungal propagules to counts exceeding 10^8. The ground impacts the microorganism counts in that there is an increase of microorganisms during storage. The surface fungi that are increased by touching the ground are not responsible for the internal rot.

Since some of the fungi found in chestnut produce mycotoxins, there may be an assumption that mycotoxins would be found in chestnuts. Mycotoxins, secondary metabolites produced by fungi, primarily are found on grains, peanuts and other commodities. There are three types of mycotoxins: deoxynivalenol (DON); ochratoxins; and, zearalenones. Just because a species of mycotoxin-producing fungus is present in a chestnut does not mean that mycotoxins are formed. There can be ramification of tissue by mycotoxin-producing fungi that do not yield mycotoxins while a small amount of other fungi yield copious amounts of mycotoxins. Something is turning off and on mycotoxin production. Even if they cannot see the fungus growing, they can detect amounts of DON in chestnuts. They are trying to reduce those fungi, as they found the amount of DON in 'Colossal' skyrockets after 90 days of storage. A selection of Chinese chestnut, 'Everfresh' (which rarely decays), does not produce DON. With respect to ocratoxin, 'Colossal' also surpasses the recommended amount sometime in Janaury (assuming storage beginning in October). Mycotoxin amounts can be reduced by using cultivar selections. 'Colossal' does not approach the recommended amount of zearalenones found to be hazardous. Fulbright discussed ways to reduce post-harvest mold. One way is to mix Euro/Japanese hybrids (that have significant mold problems) with cultivars that have fewer problems.

Swollen/superficial cankers. Fulbright showed chestnut stems with swollen/superficial cankers and he asked the group, 'what causes swellings such as these?' Many in the group responded, hypovirus. The stems he showed were part of the irradiation study planted in Michigan. Fulbright reminded the group of the disease triangle, the host, fungus and environment. The stems that he showed will die because they do not have sufficient resistance to keep them alive. The County Line site was sufficient to keep the trees alive. If we think of hypovirulence as just the reduction of aggressiveness, then we are not thinking of biocontrol. If we think about swollen cankers, we aren't thinking about biocontrol. It is everything together that gives us biocontrol. About 10-15% of the cankers from the irradiated trees have swollen cankers, but upon sampling, most of the cankers yield virulent isolates. There is a low level of resistance that comes through the irradiated seed. Inoculations into these trees with Ep155 will result in callus tissue formation. If you couple resistance in the irradiated trees with low levels of virulence

supplied by hypovirulence, trees will live. Fulbright indicated that he believes that hypovirulence might be selecting trees that have a 'leg up' on resistance and thus they continue to grow. Hypovirulence probably will not work with the most susceptible members of any population. At County Line, there are small trees that continue to survive each year. In the irradiated population of trees, very small sprouts do not react to hypovirulence; they will die. Stems must be a little larger to begin to produce callus and swell.

Sandra Anagnostakis—Connecticut Agricultural Experiment Station

Back-crossed trees. Four families of back-crossed trees with resistance from Chinese chestnut (BC_2s) and from Japanese chestnut (BC_3s) were planted in three forest clearcuts and an old tobacco field. The last American parents in the crosses were either from Roxbury, CT or Watertown, NY. The trees are now 11 years old. The four families of trees all had larger dhbs in the field plantings than in the forest, but survival was better for all families in the forest sites. No hypovirulent isolates were applied to the test trees, but hypovirulence was used on surrounding trees to help keep them alive so that they could cross with the BC trees. There were differences in soil pH; the field planting was 5.5 while the clearcuts were 3.6 to 4.5.

F_2s in Forest Clearcuts. F_2 plantings of back-crossed trees (BC_2 trees with Chinese resistance [females] were crossed with BC_3 trees with Japanese resistance [males] from the above planting) were made in two CT forest clearcuts in 2006 and 2007. She had the help of four senior state foresters. Growth and survival have been excellent on a well-drained slope (Goodwin State Forest) and poor in a site with poor drainage (Farmington Town Forest). Both sites have heavy deer pressure and the slash left after cutting has helped protect the trees from browse.

Genetics of the *Fagaceae* Project. A 2006 cross of 'Mahogany'[female] x 'Nanking' [male] (Chinese x Chinese) yielded 309 nuts which were planted in the greenhouse in February, and 253 which were outplanted at the Experiment Station in May. Scarlet clover was planted as a cover crop (based on Jimmy Maddox's recommendation) and $2ft^2$ pieces of cardboard were placed around each tree for weed suppression. Leaf samples were sent to Tom Kubisiak for DNA testing. There also were 58 and 77 nuts from a reciprocal cross of two trees that are 'Mahogany' x two different American trees. These were grown in the greenhouse, sampled for Kubisiak and sent to Meadowview for outplanting.

International Chestnut Workshop in Bursa, Turkey. This workshop was held in October 2007. The majority of the group was from Italy and Turkey. This meeting concentrated on nut production. At the end of the meeting, there was a tour of the Kavkaz factory that produces chestnut products. The factory uses very large numbers of chestnuts.

Nutrients in Chestnuts. Nuts from 10 crosses were sent to a commercial laboratory for nutrient analyses (one analysis was $100). Four pollen parents ('Okei', 'Benton Harbor', 'Eaton' and 'Nevada') were crossed with 'Colossal' in Michigan (with the help of Dennis Fulbright). One parent ('Okei') was crossed with 'Colossal' in CT. Pollen parents 'Lockwood', 'Little Giant', 'Essate Jap', and two American chestnuts were used on a promising hybrid in CT. The pollen parent made little difference in fiber, protein or fat content of nuts from 'Colossal' and the nuts yielded in MI and CT using 'Okei' as the pollen parent had similar content of these nutrients. Fat content was lower in 'Colossal' nuts than in the CT hybrid nuts and protein and fat content varied with pollen parent in nuts from the CT hybrid. Fatty acid contents in the nuts from the CT hybrid and from 'Colossal' were different depending on which pollen parent was used. It was suggested that it is the oleic acid content of American chestnuts that makes them taste sweeter than other chestnuts and low linoleic acid content has been linked to better storage properties. If the pollen parent affects the nutrient contents of the nuts, orchard designers should take this into account.

Hammond Tree. The Hammond tree in Syosset, Long Island was used by Arthur Graves in 1930 to produce many hybrids including the 'Hammond-86' which was his favorite. The Hammond tree is about 110 years old. There are two sides on the tree (east and west). Graves felt the east side of the tree was of hybrid origin and the west side was pure Japanese. A branch on the west side was broken during Hurricane Gloria. The west side of the tree yields three nuts per bur and is peroxidase BB. The east side, probably a European x Japanese hybrid, yields one nut per bur and is peroxidase AB.

Michele R. Warmund—University of Missouri-Columbia

Flowering Sequence. Primary burs (1^o) are formed, in Missouri, by 30 June and secondary (2^o) burs are formed by late July or early August. Primary burs are generally responsible for the crop of nuts. In trying to balance crop loads in Chinese chestnut, Warmund has been frustrated with the flowering habit of chestnut. The whole reason for this is nut size. The following scale was developed:

Special	≥38 mm
Extra large	35-38 mm
Large	32-35 mm
Standard	29-31 mm
Medium	25-29 mm
Small	<25 mm

There was substantial rain in Missouri in 2008 and their nut size has been tremendous. This made it very apparent that they are vastly under-irrigating their chestnut plantings. The disadvantages of 2^o burs are: limb breakage, reduced 1^o bur weight at harvest and an adverse effect on subsequent 1^o bur production. 'Willamette' is one of the worst cultivars for 2^o burs. They often have as many as thirty 2^o burs per shoot. They tried hand removal of 2^o burs; this is not something you would typically recommend to a grower. In an experiment conducted in 2006 and 2007, hand removal of 2^o burs resulted in larger nut weight from the 1^o burs. Hand removal of 2^o burs resulted in increased number of 1^o burs that are set the following year. Since hand removal is not practical, they assessed chemical treatments: Accel; NAA; Sevin; and, NAA + Sevin. Accel is labeled for pistachio. None of the treatments worked. Several years ago, the Chinese reported a mysterious compound, 'SXC' that they used to remove primary flowers from Chinese chestnut. This was sprayed in early June on whole trees. Warmund has been trying to find out what SXC is composed of. It turned out to be a gametocide, developed by Monsanto, under the trade name, *Genesis*. This was used to induce male sterility in wheat. It is no longer manufactured. What should they do in the meantime to combat 2^o burs? They need to look at cultivars that do not produce a lot of 2^o burs. Of the 75 cultivars that they have, the majority are heavy producers of 2^o burs. The cultivars that have few 2^o burs are: (1) AU-Homestead; (2) Peach; (3) Simpson; (4) Carr; (5) Qing; and, (6) Gideon. In an event that produced very cold temperatures (18F) in the spring, all of the 2^o burs were killed, but the cultivar, 'Willamette' produced a marketable crop of chestnut as a result of the freeze. Warmund commented that in areas where late spring frost may be common; thus, Midwestern chestnut growers may want to consider cultivars that have abundant 2^o burs as they may be able to harvest a marketable crop. What are conditions that limit 2^o burs formation? Is it light, temperature, average daily temperature?

Little Giant and Other Chestnut Cultivars as a Source of Genetic Dwarfing for Chinese Chestnut Trees. In March 2006, chestnuts obtained from the Connecticut Agricultural Experiment Station were sown to produce rootstocks. These chestnuts included: 'King Arthur', 'Little Giant', and 'Hope'. These seedlings were used as rootstock and grafted with 'Auburn Super' and 'Eaton' in April 2008. Trees will be field planted in spring 2009 and evaluated for a 10 year period. Generally, marketable yields are expected after the fourth growing season.

Other rootstock trials in progress include 12 replications of each of the following combinations field planted in 2006: Eaton/Cropper, Eaton/Little Giant/Cropper, Auburn Super/Cropper, Auburn Super /Little Giant/ Cropper, Eaton/Little Giant, and Cropper/Little Giant. 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*: In the 2006 trial, mean fruit weight per tree was:

AU-Super/Cropper	297 g (11 trees)
AU-Super/ Little Giant	279 g (2 trees)
AU-Super/ LG/ Cropper	272 g (3 trees)
Eaton/ Cropper	65 g (5 trees)
Eaton/LG/Cropper	18 g (1 tree)
Eaton/Little Giant	0 g

Increasing Chinese Chestnut Primary Nut Weight and Bur Production by Hand Removal of Secondary Burs. 'Orrin' and 'Willamette' Chinese chestnut trees growing at the HARC were used for this experiment. The following three treatments were imposed on shoots of chestnut trees in 2006: (1) hand removal of 2^o burs from shoots bearing both 1^o and 2^o burs (H); (2) 2^o burs not removed on shoots bearing 1^o and 2^o burs; and, (3) labeling shoots bearing 1^o burs but no 2^o burs. Treatments were repeated on the original shoots in 2007. *Key Findings and Outcomes*: Removal of 2^o burs enhanced 1^o nut weight per shoot at harvest and the number of shoots bearing 1^o burs in the subsequent growing season on 'Orrin' trees. After a 2007 spring freeze that eliminated 1^o burs, 'Willamette' trees produced a marketable crop from 2^o burs.

Characterization of Secondary Flowering and Fruiting of 'Willamette' Chinese Chestnut Trees. In 2007 and 2008, the number of 2^o burs produced and retained on 'Willamette' trees were recorded at weekly intervals from 1 Aug. to 1 Nov. Shoots on each of 5 to 10 trees tree were tagged with either no 1^o burs or one 1^o bur. In 2008, shoots with either one or two 1^o burs were labeled. Hourly temperatures, rainfall, and solar radiation were also recorded during this period. Primary and secondary fruit weights per shoot were recorded at harvest. Periods of secondary bur abortion and fruit set will be correlated with environmental conditions. Additionally, a rating scale of 0 (no burs) to 5 (100% of shoots with burs) was used to evaluate secondary bur production on trees of each cultivar in the Chinese chestnut repository in Sept. 2008. *Key Findings and Outcomes*: In 2007, as many as 30, 2^o burs per shoot were produced from 26 July to 16 Sept. The greatest number of 2^o burs were recorded on 16 Sept. Shoots with one 1o bur primary bur per shoot had fewer 2^o burs (7.7) than those with no 1^o burs. The greatest loss of 2^o burs occurred between 23 and 30 Sept. In the repository, cultivars with numerous 2^o burs (50-75%) included 'Armstrong', 'Eaton', 'Miller 72-76', 'AU-Cropper', 'Ford's Tall', 'Mossbarger', 'Carolina', 'Jersey Gem', 'Revival', 'Crane', 'Orrin', and 'Willamette'. Cultivars with few 2^o burs were 'AU-Homestead', 'Peach ', 'Simpson', 'Carr', 'Qing', and 'Gideon'. Anagnostakis commented that she sees no 2^o burs in CT unless it is very wet in the spring.

Chip Budding of 'AU-Super' Chinese Chestnut Scions on AU-Cropper and Qing Seedling Rootstocks. A study was conducted to ascertain the anatomical structure of grafted and non-grafted 'Qing' and 'AU-Super' Chinese chestnut stems, as well as the optimal time of chip budding 'AU-Super' on 'Qing' and 'AU-Cropper' seedling rootstocks. Twenty trees of each scion/rootstock combination were budded on 21 July, 15 Aug., and 1 and 19 Sept., 2006. Diameters of the rootstock liners were measured at the time of budding. Scion bud diameters were recorded on 23 Mar. before growth was visible and unions were evaluated on 25 Apr. 2007. Sectioning and examination of tissue is in progress. *Key Findings and Outcomes*: Budding success increased from 15% on 21 July to 65% and 75% for 'Qing' and 'AU-Cropper' rootstocks, respectively, on 19 Sept. Mean scion bud diameters of trees that

formed successful unions and produced scion bud growth in the spring were generally greater than those of trees that did not produce scion bud growth in the spring. For 'AU-Cropper', mean scion bud diameters of successful grafts were ≥4.1 mm, while those of 'Qing' were >3.6 mm. At the first two budding dates, tissue growth around the budding tape was excessive and sometimes covered the scion bud. At all budding dates, 75 to 100% of the budded trees produced callus around the bud plate in the fall, but in the spring, scion buds often failed to grow on trees with good callus formation. Mean rootstock diameter generally increased at successive budding dates. However, rootstock diameter did not appear to influence percent bud take. Warmund commented that low moisture content is needed in the stems. Rootstocks should be brought in on St. Patrick's Day and they should be given no water as a low moisture content is needed prior to grafting. After budding, the water should be added slowly in a shaded environment. Warmund commented that whip and tongue grafts can give 95% success rate for grafts done in the spring.

Specialty Nuts Germplasm Committee. This committee included specialty nut croups including chestnut, filbert and pistachio. This germplasm committee will allow access to grant funds and funding for national and international collection trips. Warmund is serving as the first chair of the committee. The University of Missouri-Columbia will serve as the repository for Chinese chestnut. Warmund asked Ken Hunt to put together a list of public chestnut growers (and some private) for entry into GRIN (Genetic Resources Information Network). This database will allow growers to see what germplasm is available. Warmund set an ambitious goal; she would like to develop an Excel spreadsheet that list species and cultivars held by researchers/growers. There is a need to gather information from sites with chestnut collections (cv., species, origin, discoverer, year). Grants available for germplasm evaluation and collection were due October 20, 2008.

Carmen Medina-Ferrer—Michigan State University

Understanding pollination in Michigan orchards. The problem in Michigan chestnut orchards is low yields. The MI chestnut industry is growing due to the need for quality chestnuts. Many MI chestnut orchards are primarily planted with the cultivar 'Colossal'. Growers utilize certain cultivars as pollinizers in their orchards. Orchardists often have questions regarding nut-producing trees that have died. When there is resprouting, the orchardists would like to know if the spouts are from the graft or the rootstock. Some individuals who inherit orchards often have no idea what cultivars have been planted. To address this problem, Medina and Fulbright have been using SSRs to help the MI orchardists identify cultivars in their orchards. Are there certain cultivars that are good pollinizers? Another challenge MI growers face is 'flat' nuts. It is their hope to better understand the pollination process to assist growers. Many pollinizers have been planted among 'Colossal' trees and there is no certainty which cultivar is responsible for pollination. The objective of her Ph.D. study is to determine which combination of cultivars growing in Michigan orchards will result in maximum pollination. Maximum yield potential will only be realized when all nuts in a bur are pollinized. Medina has observed: (1) trees in MSU-variety trials are not limited in number of nuts; (2) trees in orchards generally show limitations in the number of nuts pollinized; (3) physiological rot of nuts has unknown causes; and (4) the nutritional value of nut changes with pollen parent. Medina's goal is to improve the understanding of chestnut reproductive phenology, pollination, and fruit-set using genetic analysis of parents and progeny. Medina used 50 chestnuts collected and germinated from open-pollinated crosses. Her crosses were:

- 'Colossal' x 'Okei'
- 'Colossal' x 'Benton Harbor'
- 'Colossal' x 'Eaton'
- 'Colossal' x 'Nevada' (the 'Nevada' cultivar does not do well in the cold climate of Michigan)

DNA was collected from the 50 nuts from each cross; for the DNA fingerprinting, she used simple sequence repeats (SSRs) or microsatellites. The universal primer was $(GTG)_5$ and the specific primers were: CsCAT1; CsCAT16; EMCs15; EMCs3; and, ssrQrZAG96. She is still in the process of generating data from 2007. She would like to have a list of the loci she is testing and determine the amount of alleles per locus and their level of heterozygocity. In the long-term, she hopes to characterize and provide for each cultivar grown in MI, a fingerprint from each locus.

Medina listed the challenges of pollination in orchards:

1. Is pollen/flower timing critical? (What cultivar is pollinizing early or late?) (How efficient are the pollinizers with the time of flower receptivity?)
2. Pollen incompatibility—are there some trees that produce pollen but never pollinize certain trees? (Can Chinese cultivars efficiently pollinate European cultivars in an orchard?) (Does incompatibility affect fruit-set, at pollination, fertilization or during the development of the embryo?)
3. How much pollen is needed? (Is the amount of pollen available in an orchard sufficient to pollinize all of the flowers?) (Is there competition for pollen, compatible versus incompatible?)

In 2008, she chose an isolated orchard and conducted a controlled pollination study. She identified the primary flowers and looked at timing when stigma were fully developed. If we have a pollen source, does it matter how many times the flower is visited by a pollen grain? Does that affect the end product? She defined eight treatments where pollen was applied at different times (1=June 24; 2=July 7; 3=July 15), as shown below:

	Pollen application (time)		
Treatment	1	2	3
A	+	+	+
B	-	-	-
C	+	-	-
D	-	+	-
E	-	-	+
F	+	+	-
G	-	+	+
H	+	-	+

Pollen applications were either single events (treatments C, D and E), double events (treatments F, G and H), triple events (treatment A) or no pollination (treatment B). She used 'Benton Harbor' catkins because she wanted progeny that could be differentiated using her genetic markers. A minimum of 21 flowers were pollinated per treatment. She cut catkins into 1 inch segments and used 60 segments per treatment. Medina's hypothesis is that three pollen applications will result in a higher number of nuts per bur. Her results were as follows:

Treatment	Pollen applications (time) 1	2	3	burs	Total number of nuts	possible nuts	Healthy nuts %
A	+	+	+	15	7, 1, 1	46	22
B	-	-	-	13	0, 0, 0	39	
C	+	-	-	13	0, 0, 0	42	
D	-	+	-	30	6, 3, 2	90	6
E	-	-	+	25	0, 0, 0	75	
F	+	+	-	12	0, 1, 0	37	
G	-	+	+	20	6, 1, 3	62	9
H	+	-	+	13	0, 0, 0	39	
Not pollinated	-	-	-	52	2, 0, 2	163	1

In a chestnut orchard located in Maple City, MI, 10 year old trees in this orchard produce many flowers but no nuts. Medina wants to test the hypothesis that in a mixed population of two pollen genotypes, and one flower genotype, the pollen type with the highest number of mature pollen grains will have the highest frequency on the genotypes of the F_1 generation. The mixtures of pollen are listed below:

Treatment	Pollen Genotype (cultivar)	Pollen ratio (%)
A	Benton Harbor	100
B	Benton Harbor + Okei	25/75
C	Benton Harbor + Okei	50/50
D	Benton Harbor + Okei	75/25
E	Okei	100
F	Control	0

Medina used 60 flowers per treatment. Nuts from this trial had not been collected as of the NE-1033 meeting.

Pam Kazmierczak, UC-Davis

Chestnut inventory. Rootstock material from Lucienne Gunder was 't-budded'. The grafted cultivars at the Foundation Plant Services/UC-Davis are as follows:

- 4 'Marrone Comballe'
- 2 'Marrone di Chusa Pesio'
- 2 'Marrone di Marradi'
- 1 'De Coppi'
- 1 'Qing'

- 2 ‘Luvall’s Monster’
- 4 ‘Easton’
- 4 ‘Campell, NC’

These trees will be outplanted in 2009. These are original imports to California from 2004 and are contained within UC-Davis Foundation Plant Services. They were inspected this fall by the California Department of Food and Agriculture. They are scheduled for release in the spring of 2009. As a note, Lucienne Gunder sold her nursery.

Fred Hebard—The American Chestnut Foundation, Meadowview

China trip. The objectives of the trip to China for the 2008 IV International Chestnut Conferences were to:

- Seek collaboration in planting and testing of TACF breeding products in China
- Observe the blight fungus in its native environment
- Document the basic ecology of wild Chinese chestnut populations

The group arrived in Beijing on September 10, and flew to Yichang, Hubei Province for several days in the Dalaoling Natural Reserve. They traveled by train to Ankang, Shaanxi Province and spent several days studying chestnut in Ankang. On September 20, they drove through the Qinling mountains to Xi’an and toured the Terracotta Army. On September 23, they flew back to Beijing and toured the Great Wall, were debriefed in the U.S. Embassy, and had meetings in Beijing Forest University and Peking University. The trip concluded on September 25.

With regard to Chinese *Castanea* species, *Castanea mollissima* is the most widely distributed among the three native chestnut species in China, ranging from 17-41ºN and 91-126ºE. It has 2-3 nuts per bur typically. *Castanea henryi* has the narrowest distribution among the three native chestnut species in China, ranging from 22-32ºN and 103-122ºE. It has one nut per bur typically. The spatial distribution of *C. seguinii* is more similar to *C. mollissima*, ranging from 21-41ºN and 102-123ºE. It has 2-3 nuts per bur typically.

Preliminary conclusions from the trip were:

- *Cryphonectria* was confirmed by isolation from *Castanea mollissima* and *C. henryi*, and cankers or lesions that appeared to be blight were common on all three species (this would have to be confirmed by systematic isolation). However, the cambium appeared to be generally uninjured by these lesions, and trees were thriving despite the presence of the disease.
- *Castanea mollissima* in native forest habitats was somewhat more tree-like than regarded in the United States.
- *Castanea henryi* is the largest and most forest-tree-like of the three native chestnuts, but all became timber trees. *C. seguinii* was larger than is reported in the literature.

Potential collaborators at were identified at two sites.

Backcrossing. The backcross technique was first begun with American chestnut in 1989. First, American and Chinese chestnut trees are crossed with each other. The progeny from this first cross are then backcrossed to American chestnut. Each cycle of backcrossing reduces the fraction of Chinese genes by a factor of one half. Blight resistance is retained by inoculating progeny and selecting resistant individuals. BC_3s were intercrossed in 1999 and the progeny from BC_3s were gathered in 2005. More than one line of American chestnut is needed; the Meadowview farm is using 20 lines to avoid inbreeding. Also, more than one source of blight resistance from *C. mollissima* is being used. Currently, there are 49,603 trees at Meadowview. Nearly 3,900 B_3F_3 nuts were harvested in 2008. In the intercrossing of the third backcrosses, the B_3-F_2 family size needs to be 9 or 10 to capture most alleles

from each B_3. Currently, Hebard does not use molecular markers for selection; he still relies on morphological markers. Hebard's selection for recurrent type morphological traits includes:

- Trichome density and type
- Stipule size and shape
- Leaf shape
- Tooth shape
- Lenticel density, size and color
- Stem color
- Form
- Time of leaf emergence
- Vigor

Hebard uses two virulent *C. parasitica* isolates to screen trees: EP155 (high end of the virulence scale) and SG1 2-3 (low end of the virulence scale). Hebard feels that he can make a qualitative distinction using both isolates.

Hebard commented that resistance is not holding up as well as he thought. Environmental factors can have a large effect on tree condition. Hebard showed slides of their best B_3-F_2 from the first season of inoculation, in 2004. Aside from the form, blight resistance, at this point, is awful. What's going on? Is the breeding program in trouble? No, the breeding program is not in critical trouble; they are improving the blight resistance of the American chestnut tree. Growing American chestnut trees the size of the second backcross is not possible (without hypovirulence, which was not a factor here). Second backcross F_2s and F_3s have even better resistance.

In Chinese chestnut, the phelloderm becomes lignified. This facilitates the sloughing of cankers. Lignification of wound periderm phelloderm occurs in Chinese but not American chestnut. It could be an important component of blight resistance, reducing penetration of wound periderm and facilitating sloughing of cankers. We may not be selecting for it with our current screen for blight resistance. Hebard plans to test these hypotheses shortly.

Other initiatives at Meadowview include:

- Develop a rapid screen for blight resistance
- Use the screen to test for resistance-breaking strains of the blight fungus and to uncover the genetics of virulence in the fungus
- Use the screen to evaluate more progeny for blight resistance than is currently possible
- Uncover the genetics of blight resistance in chestnut, to increase the number of sources of blight resistance in a rational manner, rather than using a shotgun approach
- Establish a provenance test of chestnut
- Evaluate the combination of hypovirulence and resistance for improved control of blight
- Evaluate progeny for genetic abnormalities

Leila Pinchot, University of Tennessee, Knoxville

Containerized chestnut shadehouse study (in cooperation with Stacy Clark, USFS Southern Research Station). The objective of this study was to study the effects of light on early growth and development on American chestnut seedlings germinated in 10-gallon 'spin out' coated pots. The study was conducted at the East Tennessee research and education center in Knoxville. The research was conducted on two TACF BC_3F_3 families to study the effect of three levels of light on early seedling growth and development. Twelve 10' x 10' plots, located in full sun, were established on a straight transect. Plots represented four replications each of 0% shade, 30% and 70% shade. One-hundred, sixty-two chestnut seeds were planted in 10-gallon pots with hardwood mix and coated with 'spin-out' to

prevent root binding. Once the chestnuts germinated, pots were relocated to the twelve plots, with representatives from both BC_3F_3 families located in each plot. Chestnut seedlings were watered as necessary and fertilized in mid-May. UT personnel measured photosynthetic activity of four seedlings per plot in last August using a LI-COR photosynthesis measurement system. Photosynthetically active radiation (PAR) was measured using an Accu-Par ceptometer. PAR measurements were taken within and outside of the shadehouses in order to determine available light for each of the treatments. They found that the shadehouse values were not accurate. The 30% shadehouse was actually 40% and the 70% shadehouse was actually 60%. Data on this experiment is forthcoming.

Chestnut morphology study (in cooperation with Fred Hebard, TACF, Sandra Anagnostakis, CAES, The American Chestnut Cooperator's Foundation, Georgia Forestry Commission and the Flint River Nursery). The objective of this study is to compare the morphological traits of the ACCF's American, TACF's American, Chinese, F_1, BC_3F_3, BC_2F_3 and BC_1F_1 and CAES' Japanese, BC_1F_1 and BC_2F_1 chestnut seedlings in order to determine how closely later generations of backcross chestnuts resemble American chestnuts. Nuts from approximately 20 families representing American, Chinese, Japanese, F_1, BC_1F_1, BC_2F_1, BC_2F_3 and BC_3F_2 chestnut were planted at the Flint River Nursery in Georgia in January, 2008. Seedlings were fertilized and irrigated according to prescriptions developed by Paul Kormanic. In mid-September 2008, numerous twig and bud characteristics were evaluated on site for five individuals from each family. Characteristics included stem color, density of stem hairs, presence/absence of stipules, bud color, bud shape, frequency of bud hairs and other. One leaf from each sampled individual was removed and stored in a plant press for later evaluation. Numerous characteristics, such as hairs on underside of leaf, leaf shape, teeth hooking and others will be evaluated. Backcross chestnuts will be compared with pure American, Chinese (TACF material) and Japanese (CAES material) chestnut.

Daniel Boone National Forest Silvicultural Study (in cooperation with USDA Southern Research Station, Daniel Boone National Forest, TACF). The objective of this study was to test the effects of three silvicultural treatments on growth, survival and competitive ability of planted BC_2F_3 seedlings. The long-term goal is to provide forest managers with appropriate silvicultural prescriptions for American chestnut reintroduction once blight-resistant seedlings become available. Approximately 500 TACF BC_2F_3 chestnut seedlings will be planted with American and Chinese chestnut seedlings under three silvicultural treatments at the Daniel Boone National Forest in the spring of 2009. The three treatments represent a continuum of light levels, from high light in a two-aged shelterwood with reserves, to moderate light in a thinning prescription, to low light in an oak shelterwood. Seedling survival, height growth, root collar diameter and light available to each seedling will be measured.

Field testing of blight resistant B_3F_3 chestnuts (in coopeartion with Stacy Clark, USFS Southern Research Station). The objective of this study is to evaluate TACF BC_3F_3 chestnut material in the field including growth performance, survival, morphological characteristics and blight resistance in order to define critical silvicultural parameters for successful restoration. In the spring of 2009, approximately 80 American, 60 Chinese, 60 BC_1F_3, 60 BC_2F_3, and 100 BC_3F_1 will be planted in 2 plots on each of three National Forests (George Washington and Thomas Jefferson National Forest in Virginia, Cherokee National Forest in Tennessee and the Nantahala National Forest in North Carolina). Plantings will be implemented as enrichment plantings within existing 15-40 acre shelterwood with reserves harvest units treated within a year prior to planting. Seedlings will be planted in a replicated experimental design so that the differences in seedling performances among breeding generation and genetic family within breeding generation can be determined. Annual evaluations on seedling performance will be conducted for the first three years on survival, height and basal diameter growth, apical dominance rating, competitive ability, canker severity from chestnut blight and presence of other

damaging biotic and abiotic agents. After planting, measurements will be taken at 3, 5 and 10 years. Additionally, site-specific measurements will include amount of competition at each site and site characteristic measurements (slope, aspect, soil type).

John Carlson, Pennsylvania State University

PA-TACF activities (reporting for Sara Fitzsimmons, Regional Science Coordinator, TACF). The PA Chapter is continuing work on many already established initiatives. Investigations into the use of cytoplasmic male sterility, various silvicultural methods, biomass and coppicing plantings, and plantings of various generations of breeding material continued in full force. Though there were no major breakthroughs throughout this past year, each project continues to see progress.

One of the most exciting developments in Pennsylvania was the harvesting of the first B_3F_3 generation seed to be produced in the state. These seed were collected from Meadowview (TACF's main breeding farm in southwestern VA) derived material. The seed were distributed to two locations for testing purposes. Over 100 seed were planted in containers for the purpose of small-stem inoculation. Another 100 seed were distributed to the Thorpewood Foundation in Thurmont, MD for field planting. Initial survival of these plantings is good. Inoculations on greenhouse-grown material will likely take place in early summer of 2009.

Once again, thousands of seed were planted across the state. At the Penn State Arboretum, almost 2,000 seed were planted as gypsy moth larvae rained upon the volunteers. A new initiative with the Lancaster County Conservancy, the National Wild Turkey Federation, and the PA Game Commission saw a planting of almost 3,000 seed at the House Rock planting in Lancaster County, just up from the Susquehanna River. That planting was installed as part of initial restoration initiatives of the LCC and PA-TACF, looking at several advanced hybrid generations to observe their suitability for long-term restoration goals. B_1F_3, B_2F_3, and B_3F_2 generations were all planted together to observe differences in growth, form, and resistance between those generations.

The Chapter worked to inoculate several hundred trees of the B_3F_2 generation at two locations. At both locations, the Penn State Arboretum and Blair Carbaugh's orchard in Danville, PA, volunteers performed inoculations on the first members of material derived from local Pennsylvania stock. It is expected that these trees will begin to produce progeny in the fall of 2009. Several other locations with fourth generation (BC_3) material also were inoculated across the state.

One exciting find by a Chapter member in northwest Pennsylvania was an orchard that was originally established by Mr. Haun in the early 1980s. This orchard, comprised exclusively of American chestnuts (no hybrids), was cared for by Mr. Haun until his death in the mid-1980s. Since this, his son had not managed the chestnuts, but he also did not cut down the trees. In 2007, a student from Grove City College took several initial measurements on the planting. Of an original planting of 500 trees, 250 still stand. As to be expected, none of these trees are original stems, but the subsequent clumps appear to be doing quite well. These trees offer a chance to investigate the life of an American chestnut orchard faced with incredible amounts of blight infection. These trees also demonstrate the value of planting trees with little to no blight-resistance. Besides conserving local genetic reserves, these trees offer great reservoirs of seed production, are an attraction to wildlife, and offer easily accessible breeding material.

Notes From China Trip, September 12-24, 2008 (reporting for Kim Steiner, School of Forest Resources, Penn State University). Representatives of The American Chestnut Foundation were hosted by Chinese researchers and officials on a unique tour of natural forest chestnut stands and nut production orchards in China prior to the 4th International Chestnut Symposium in Beijing. The "tour group" included Kim Steiner of Penn State University, Fred Hebard of The American Chestnut Foundation, Sara Fitzsimmons, Regional Science Coordinator for TACF, Songlin Fei of the University of Kentucky, and

Fred Paillet, retired USGS geologist. Sara Fitzsimmons blog on this trip can be found at: http://www.personal.psu.edu/sff3/blogs/chestnuts_in_china/

Preliminary conclusions:

- *Cryphonectria* was confirmed by isolation on *Castanea mollissima* and *C. henryi*, and cankers or lesions that appeared to be blight were common on all three species (this would have to be confirmed by systematic isolation). However, the cambium appeared to be generally uninjured by these lesions, and trees were thriving despite the presence of the disease.
- *Castanea mollissima* in native forest habitats was somewhat more tree-like than we regard it in the United States.
- *Castanea henryi* is the largest and most forest-tree-like of the three native chestnuts, but all became timber trees. *C. seguinii* was larger than is reported in the literature.
- Potential collaborators at Dalaoling are Dr. Shen Zehao, Dr. He Wei, and Mr. Xu Shen-Dong (who seems eager to raise the status of his forest preserve).
- Potential collaborators at Ankang (Nan Gongshan National Park) are Dr. Lu Zhoumin, Mr. Chen, and Mr. Liao.

Mike Marshall, Shippensburg University

Inoculations of *Cryphonectria parasitica* on various fruits and vegetables. Previous researchers have employed apples of several varieties to assess virulence of *Cryphonectria parasitica* and *C. cubensis* isolates. Curiosity on the virulence of *C. parasitica* on other fruits and vegetables led to the inoculation of the following: apple (*Malus domestica*),vars. Gala, Gold Delicious, and Granny Smith; avocados (*Persea Americana*); Chayote squash (*Sechium edule*); Gold tamarillo (*Solanum betaceum*); grapes (*Vitis vinifera*), seedless white and purple; grapefruit (*Citrus x paradisi*), red; lychee fruit (*Litchi chinensis*); nectarine (*Prunus persica*); orange, (*Citrus sinensis*) naval; Patty Pan squash (*Cucurbita pepo*); pears (*Pyrus communis*), vars. Bartlet and D'anjou; Pepino Melon (*Solanum muricatum*); plum (*Prunus domestica*); pomegranate (*Punicia granatum*); Prickly Pear cactus fruit (*Opunta spp*.); star fruit (*Averrhoa carambola*); strawberry (*Fragaria x ananassa*); and tomatillos (*Physalis philadelphica*). Several vegetables also were inoculated: broccoli (*Brassica oleracea*), stem section; carrots (*Daucus carota*); celery (*Apium graveolens*) stalk section; ginger root (*Zingaber officianale*); potato (*Solanum tuberosum*) tuber; and string beans (*Phaseolus vulgaris*) pods.

Small agar plugs of PDA cultures of two virulent *C. parasitica* isolates, Bockenhauer (MAT-2) and Schomberg (MAT-1), were inserted into shallow cork borer holes (4 mm i.d., 2 cm apart) made in the plant specimens listed above. Fruits were selected that were blemish-free. All plant material was first washed with "Dawn" (Proctor & Gamble) antibacterial soap (containing Triclosan), rinsed in tap water, and blotted on a clean paper towel. Total surface sterilization was not done, but the inoculation sites were swabbed with 70% propanol before the cork borer wounds were made. Each inoculated specimen was then placed in an appropriately sized (760 or 190 ml) plastic container (Rubbermaid "Takealongs") with a snap-on lid that had been similarly washed and swabbed out with 70% propanol; the lids were left unsnapped. All inoculated material was incubated at 27^{o}C with a16 hr light / 8 hr dark schedule for 17 days before the results were photographed.

Cryphonectria parasitica was able to grow on most of the inoculated materials, the potato tubers being a notable exception. Growth on most of the vegetable material, much of which had dried out excessively, was minimal. The best growth and sporulation was seen on strawberries, the pear varieties, pepino melon, pomegranate, and avocado, in that order. Work is ongoing to explore the suitability of media made from these sources for general *C. parasitica* culture. The inability of *C. parasitica* to grow on potato tuber pieces also is being explored further.

Paul Sisco, The American Chestnut Foundation, Southern Appalachian Regional Science Coordinator

Father tree program: Year Two. This summer volunteers from the Southern Chapters were again able to bring pollen from their hard-to-access American chestnut trees to Meadowview. Cooperation among chapters was excellent, so that every chapter did not need to have helpers there all the time. Alabama, the Carolinas, Georgia, Kentucky and Tennessee were all represented.

Southern Regional Meeting. Because of a conflict with the special TACF chapter meeting in Virginia called by Dick Will, it was decided not to have a Southern Regional Meeting in February, 2008. The next scheduled meeting is February, 2009. These region-wide meetings have been very useful in sharing information about the particular scientific problems of the south – chestnut/chinkapin introgression, and pests and pathogens such as *Phytophthora cinnamomi* and Asian Ambrosia beetle.

Southern Pests and Pathogens: Breeding for Resistance to *Phytophthora cinnamomi*. Joe James of the Carolinas Chapter and Steve Jeffers, Phytophthora expert from Clemson University, continue their multi-year experiment to determine which TACF backcross families have resistance to *Phytophthora cinnamomi*, an organism that can destroy the root systems of American chestnut trees. In the summer of 2008, James expanded his research. Surviving seedlings selected from the 2007 experiment were transplanted into pots that were kept irrigated during the summer. About 70% of those died during the summer of 2008. Those that survived the second year after inoculation will then be transplanted into Joe's farm. Seedlings that survived the 2007 Phytophthora screening were transplanted into pots for further evaluation before transplanting into the field. About 70% of them died during the summer of 2008. Conclusions from the Phytophthora work are as follows:

- The resistance from Chinese chestnut is controlled by more than one gene.
- The resistance genes are not completely dominant. F_1's have high survival but their roots have lesions.
- It may be difficult to get American-looking hybrids that have resistance to both *Phytophthora cinnamomi* and *Cryphonectria parasitica*
- Joe James' next tactic is to test more F_2's, in hopes of seeing higher levels of resistance to Phytophthora.

Southern Pests and Pathogens: Phosphites to protect seedlings against *Phytophthora cinnamomi.* Steve Barilovits of the Carolinas Chapter conducted a backyard experiment this summer to test the efficacy of various phosphite products to protect first-year chestnut seedlings against *Phytophthora cinnamomi*. Steve experimented with Aliette –WDG (aluminum phosphite), Agri-Fos (potassium phosphite), and Prudent-44 (urea phosphite). His first-year results were that both Agri-Fos and Prudent-44 provided a good level of protection, but that Aliette, at the rate he applied, was relatively ineffective. He is going to repeat the experiment next summer.

Southern Pests and Pathogens: Asian Ambrosia beetle attacks chestnut trees. The Asian Ambrosia beetle, *Xylosandrus crassiusculus*, was first found on peach trees in Charleston, SC, in 1974. It has a wide host range and causes damage by introducing pathogenic fungi into the interior of the stem. It has attacked and caused death of both Chinese and hybrid chestnut trees in several of our orchards in the South, as well as in orchards of the American Chestnut Cooperator's Foundation (ACCF).

Drought. Severe drought in the southeast was confined to a smaller area than last year, but it severely impacted upper South Carolina, northeast Georgia, and western North Carolina.

The American Chestnut Foundation Chapter Notes:

- **Kentucky:** Plantings continue on different types of reclaimed surface-mined soils as well as state and private forest lands. Victoria Willis has taken over as breeding coordinator, now that Michael French

has a full-time job planting trees on reclaimed mine land. Several chapter members volunteered to drive to Meadowview for the Father Tree pollinations. Work is underway to get all chapter orchard records into the TACF national database.

- **Tennessee:** The chapter is now producing thousands of seed and is struggling to find places to plant them all. TN-TACF acted as host for the national TACF meeting in Chattanooga. Chapter members manned booths at several local fairs and events. Joe Schibig won a DAR award for his work with chestnut. Meagan Binkley, student with Joey Shaw and Hill Craddock at UT-Chattanooga, is completing her TACF-funded project on DNA variation in chestnut and chinkapin. Bethany Baxter, another student at UTC, completed interviews with several older folks for an oral history project, also funded in part by a grant from TACF.
- **Alabama:** The Father Tree Program has proved invaluable for the Alabama chapter, since AL has few flowering mother trees and most of them are producing only pollen. The chapter is negotiating with TVA and adjacent municipalities to try to preserve the main chapter orchard on TVA land in Muscle Shoals.
- **Georgia:** Berry College installed an irrigation system in the college orchard. Mary Belle Price provided funds for a chapter intern. The GA Father Tree pollinations at Meadowview were a success. GA-TACF hosted the beginning of the 2008 Appalachian Trail chestnut hike in early March.
- **Carolinas:** The chapter has completed 20 Clapper lines using high-altitude trees (>3500' elevation). The next chapter project, started this summer, is to use pollen from lower-altitude trees in the father tree program. These families will be screened for Phytophthora resistance before planting in an effort to create a population that is resistant to both Phytophthora root rot disease and blight.

Bob Paris—The American Chestnut Foundation, Beckley

Establishment and growth of chestnut on mined lands. The TACF and its cooperators have been planting American chestnut and backcross chestnut on mined lands in four states (KY, IN, PA, and OH) since 2002. These were mostly reclaimed lands. In December 2007, they submitted a grant to the Office of Surface Mining (OSM) to observe Chinese, American, B_1F_3, B_2F_3, and B_3F_2. The OSM covers TN, KY, OH, WV, PA, and MD. He showed slides of a reclaimed area in PA at the Smith Research Farm in Jefferson County, PA. Many of the 6-year old trees were 2'-4' tall in 2008. In Muhlenberg County, KY, chestnuts were planted on fertilized, ripped, reclaimed mined land with a weathered, brown sandstone substrate. The seedlings were established by direct seeding, mulched with black plastic and protected from voles with aluminum cylinders. The 2-year old seedlings were planted in conjunction with black locust and the chestnut seedlings were somewhat chlorotic, compared to the black locust at the site. At an active strip mine in Pike County, KY, chestnut seedlings established on tail-dumped, run-of-the-mine spoil. After two seasons of growth, chlorosis was evident on most seedlings. With the exception of nitrogen-fixing species, chestnut has the ability to grow as well as, or better than other tree species over a four state area spanning the ARRI region, indicating that backcross chestnut from TACF's breeding program are suitable trees for afforestation of mined lands.

Lisa Worthen, Hardwood Tree Improvement and Regeneration Center, Purdue University (submitted report). Worthen examined 521 outcrossed American chestnut burs containing over 1000 nuts. The nuts were removed from the burs and weighed individually. The average nut weight: 3.492g (Range = 0.53 to 7.03g; SD = 1.206g). There are 453.59 grams in a pound, thus it takes about 130 chestnuts to make a pound. (Note that Chinese chestnuts are often in excess of 20g, such that when you buy a pound in the store, there are only about 20 or so nuts in the bag). Chestnuts have no endosperm and a very small, thin pellicle that covers the embryo, which was not removed before weighing the seed.

Worthen guesses that the pellicle weighs no more than 0.05 to 0.1g such that the fruit-to-seed ratio is almost 1.

Lynne Rieske-Kinney, University of Kentucky Department of Entomology (submitted report).

Rieske-Kinney continues her investigation of the Asian chestnut gall wasp, *Dryocosmus kuriphilus* (*Hymenoptera: Cynipidae*), an exotic invader that causes round, 8-15 mm diameter, greenish-red leaf and twig galls on all chestnut species, though differences in susceptibility differ among various chestnut species and varieties. The gall wasp was accidentally introduced into North America in 1974, and has become a serious pest of chestnut worldwide. Galls suppress shoot and twig growth, reduce tree vigor and wood production, and reduce fruiting and nut yield. Severe infestations can kill trees.

Chestnut hybrids that have been developed for blight-resistance are appearing in restoration programs aimed at restoring American chestnut to the landscape, and in addition to other stressors, will be interacting with expanding Asian chestnut gall wasp populations. These interactions will undoubtedly affect chestnut production and American chestnut restoration efforts in eastern North America.

The overall goal of her research has been to evaluate the geographic range expansion of *D. kuriphilus* in North America, the effects and ecological associations that have developed as *D. kuriphilus* extends its geographic range, and species-specific and varietal differences in *Castanea* susceptibility to the gall wasp, including qualitative and quantitative differences in source strength and signaling compounds. Specific objectives are: (1) evaluate population characteristics of the Asian chestnut gall wasp, *Dryocosmus kuriphilus*, and its natural enemy recruitment, near the edges of its known geographic range in North America; (2) evaluate the extent to which its *Castanea* hosts influence gallmaker success via qualitative and quantitative differences in source strength; and, (3) the extent to which its *Castanea* hosts influence qualitative and quantitative differences in plant signaling.

S.A. Enebak, Auburn University (submitted report)

Studies are planned to develop cultural, chemical and biological production strategies for the management of *Phytophthora cinnamomi* in the large-scale production of American chestnut seedlings. Cooperative research scientists include personnel from the AU Southern Forest Nursery Cooperative and the American Chestnut Foundation. Current nursery practices will be examined and areas where infection by *P. cinnamomi* could occur will be identified. Experiments planned for these include soil and water surveys conducted this fall to examine various fungicide treatments at the East Tennessee Nursery, near Cleveland, TN. Soil samples will be collected and analyzed before and after fumigation of nursery beds for various microbial activities. Sown seedlings will be examined for any adverse effects to American chestnut from the various treatments. Phytotoxicty as measured by reduced growth and deleterious effects on root growth will be measured. In conjunction, we will be testing to see if the wounding of roots from the mechanical lifting will provide an opportune site for the pathogen once out planted in an infested site. We will be testing fungicidal root dip treatments vs. non-dip treatments of roots before out planting from lifting. In addition to fungicidal treatments, soil microbiologicals (biocontrol) will be examined.

Future studies include the use *P. cinnamomi* infested soil containment pots to test the treatments against many variables that are more favorable for the pathogen. Testing under a more controlled setting will identify areas that forest tree nurseries will be able to address in their production systems. Results from this season (2008) sowing/ treatment trials will set the stage in planting and applying the recommended treatments in various nurseries for Fall 2009.

Steven Jakobi, Alfred State College (submitted report)

Potential effect of crowding on blight resistance by different chestnut species and hybrid cultivars (in cooperation with Sara Fitzsimmons, The American Chestnut Foundation). This study was designed to investigate the potential effect of crowding on susceptibility of different chestnut species and hybrids to infection by the blight fungus, *Cryphonectria parasitica*. Sixty seeds each of cultivars of Chinese chestnut ('Entriken' from Huntingdon, PA and 'Merkel' from Penn State University [PSU]); American chestnut seeds from a tree produced from irradiated nuts on Sugarloaf Mt. MD; B_2F_3 seeds (Chinese x American backcross) from PSU; and, B_3F_2 (Chinese x American backcross) from Hershey, PA were planted on the Alfred State College farm in Allegany Co., NY, on May 10, 2008. Seeds of each species/cultivar were planted 1-, 2-, or 3 ft apart in rows in a randomized design. As of Aug., 15, 2008, 79.4 percent of 'Entriken', 55 percent of 'Merkel', 50 percent of American, 63.3 percent of B2F3, and 71.7 percent of B_3F_2 nuts germinated and produced aerial stems inside 2-ft-tall plastic planting tubes. Most of the seeds that failed to germinate were deemed to be planted too deeply by student workers and these will be replanted in 2009. Seedling mortality ranged from 1.7 percent ('Entriken') to 8.3 percent (American, B_2F_3 and B_3F_2) and appeared to be due to insect damage. The 2008 growing season produced adequate and evenly-spaced rainfall, and soil quality and drainage are excellent on this slopping site. Most of the surviving seedlings are 1-2 ft tall. A 7-ft-tall deer fence was erected in November, 2008, to reduce the amount of predation by herbivores.

<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.

Sandra Anagnostakis—Connecticut Agricultural Experiment Station

Transgenic study. An experiment with Don Nuss and co-workers to introduce genetically engineered hypovirulent strains of *C. parasitica* into a natural population of the fungus was started in 1997 in a clearcut in the Meshomasic State Forest. Sprout clumps of American chestnut were marked; 25 sprout clumps were sprayed with water and 25 with a mixture of spores from transgenic strains. This was done several times between 1997-2004 and no treatments have been done since. The dbh of all sprouts >2.5 cm was measured in the winter of 2007. In the control plot, there were 12 sprouts with a total of 18 sprouts larger than 2.5 cm dbh and the dbh per clump ranged from 2.5 -7.3 cm. Only three control sprout clumps had mean dbhs greater than 4 cm. In the treated plots, there were 15 sprout clumps with a total of 27 sprouts >2.5 cm and the dbh ranged from 2.7-12.3 cm. There were 13 sprout clumps with mean dhb greater than 4 cm. The sprouts in treated clumps are surviving longer than sprouts in the control group.

Pam Kazmierczak, UC-Davis

CHV1 disrupts secretion of cryparin in *Cryphonectria parasitica*. One of the proteins that she has focused on is cryparin, a cell surface class II hydophobin. In liquid shake culture, she has found cryparin to comprise 30% of the mRNA and up to 1% of the protein. Cryparin was chosen for study because it is very abundant. When cryparin protein was deleted, it was found to be essential for eruption of the fruiting bodies of *C. parasitica* through the outer bark layer of the chestnut tree. Because of cryparin's abundance, both as mRNA and protein, Kazmierczak chose to follow it as a marker during secretion in the presence of CHV1. Previous work showed dry weight accumulation was very similar between CHV1 infected, UEP1 and EP155/2. Cryparin in whole cell lysates accumulates to a much higher degree in the unifected strain (EP155/2) as does secreted cryparin collected from the culture filtrates of infected and unifected strains. Using the techniques of subcellular fractionation from previous studies of EP802 which contains CHV1, she adapted the protocol to compare cryparin location and

accumulation in these microsomal fractions, comparing EP67 to the viral-containing strain EP802. EP67 secretes cryparin very rapidly into culture filtrate. Fresh 3-day post inoculation shake cultures of each strain were harvested and frozen. One-tenth of the fresh harvest weight was lyophilized and used to extrapolate and calculate the dry weight of the fresh material. Equal volumes of P3 (the microsomal fraction) containing equal dry weight equivalents were loaded onto 10 ml linear gradients of Ficoll-heavy water. One ml fractions were collected, washed and run on 12.5% Tris-Tricine SDS gels. Cryparin AB that had been affinity purified to the cryparin null mutant, Δ119 was used to probe the blots. Cryparin was extracted from the lyophilized mycelia from these cultures and extracted in 60% ethanol; it showed the typical pattern of cryparin accumulation. Pulse chase analysis following radio labeled cryparin showed that cryparin dwell time within CHV1 infected cells was up to 4 hours while detectable cryparin could be detected from within EP67 cells after 5 minutes. Cells were pulsed for 20 minutes with ^{35}S cysteine and harvested after various chase times. Cytoplasmic proteins were isolated, subject to PAGE and the dried gels exposed to phospho-imaging scans. No detectable cryparin remained in the cytoplasmic fraction after 5 minutes. The take home message is that cryparin is rapidly secreted into the culture fluid where it is re-bound to the cell wall—the virus-infected fungus is greatly reduced in cryparin protein accumulation. Kazmierczak transformed a cryparin null mutant with an altered copy of the cryparin gene. This construct inserted the GFP coding region into the middle of the coding region of cryparin. This allowed her to use GFP to track patterns of accumulation of cryparin in the absence and presence of CHV1. Four independent transformation events were selected for GFP expression and single copy integration, and similar patterns of expression were found in all cases. The –CHV1 transformation event shows distinct fluorescent vesicle-like bodies. GFP quantification of cytoplasmic extracts was measured by fluorimetry. WT6 is cryparin rescue strain, GFP 0-3 is the cryparin null mutant transformed with the crp:GFP fusion vector, and GFP 0.8 is the same strain infected with CHV1 via hyphal anastomosis with a CHV1 containing strain. GFP expressing construct shows typical 3-4 DPI accumulation of signal. Cells stained with FM4-64 confirm plasma membrane localization. The localization of vesicles containing cryparin/GFP fusion protein appears to fuse with plasma membrane. Calcaflour staining of septal regions of GFP 0.8, confirms GFP accumulation accumulation in septal regions. Vesicles that contain cryparin and the hypovirus are of trans-golgi origin. There are a number of papers that report viruses affecting a fungal secretory pathway.

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 was the project proposer. Other proposers were Alice C. L. Churchill and Michael G. Milgroom, Cornell University. Other participants included: 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. ***Scope of Work:*** Genomic DNA was prepared from the most widely used and best characterized *C. parasitica* strain, EP155. Expectations from JGI include the generation of approximately 400Mb of raw sequencing reads required for 8 – 10 X coverage of the estimated 40-Mb *C. parasitica* genome, sequence assembly and initial automated annotations. The annotation process was aided by the availability of two large *C. parasitica* EST libraries that have been generated by laboratories in the U. S. and China and a linkage map; annotation was aided further by comparisons with the completed genomes of the phylogenetically related fungi *Neurospora*

crassa and *Magnaporthe grisea*. ***Update on JGI Genome Sequencing Project*:** An 8.54x assembly of the *Cryphonectria parasitica* v1.0 assembly was released to the community on March 28, 2008 for initial annotation efforts.

Brief Summary:

Genome Size:	43.9 Mbp
Total Number of scaffolds:	39
Total Number of EST clusters:	8799
Total Number of trimmed ESTs	22,765

The following average properties were predicted for 11,184 genes:

Gene Length (bp)	1783.64
Transcript length (bp)	1552.35
Protein length (aa)	465.18
Exons per gene	2.96
Exon Length (bp)	524.67
Intron Length (bp)	120.08

The genes were predicted by the following methods:

EST-derived:	1286 (12%)
Based on homology:	6157 (55%)
ab initio:	3741 (33%)

The genes were validated by the following evidence:

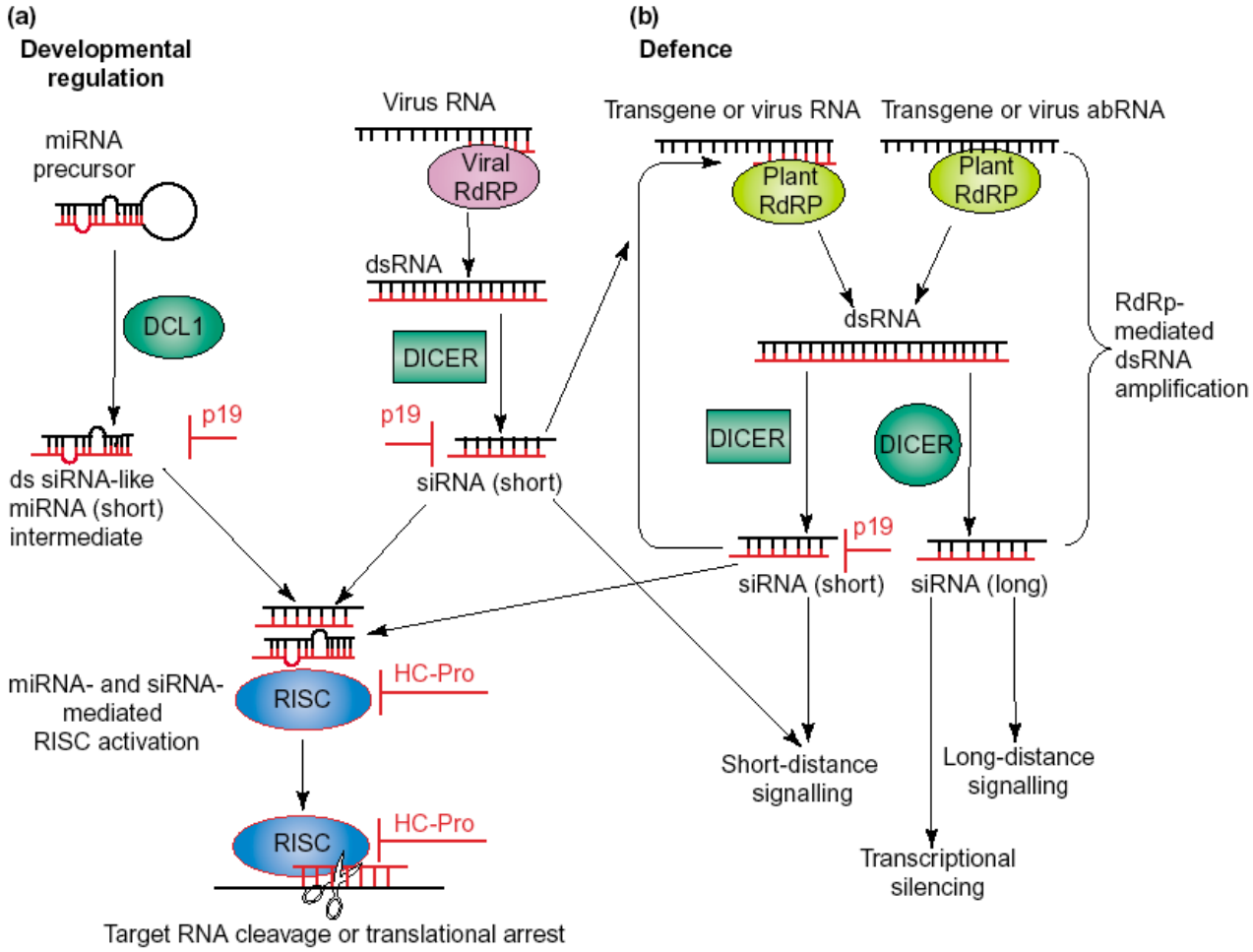
EST Support	5003 (45%)
nr protein alignments	9638 (86%)
Pfam alignments:	5588 (50%)
Complete (5'M-3'*)	9992 (89%)

The annotation team participated in annotation tutorials provided by the Joint Genome Institute on April 11, 2008 and May 9, 2008. The *C. parastica* genome assembly was released to the public on the JGI website (www.jgi.doe.gov/Cparasitica) on September 30, 2008. The annotation team held a progress report session on Sunday, October 18 following the annual NE-1033 meeting. Efforts to generate a manuscript describing the *C. parasitica* genome sequencing and assembly project are in progress.

RNA silencing as an antiviral defense mechanism in fungi. Nuss posed the question: Does *C. parasitica* have antiviral defense mechanisms to counter hypovirus modulation of fungal-plant interaction? There are two mechanisms: (1) vegetative incompatibility on a population level is one method that prevents viral spread; and, (2) RNA silencing is another method. The plant system (see diagram below) is the best characterized system but it is a complex system. He compared the plant system to what has been found in fungi. There are two things going on in plant developmental regulation that involves the RNA silencing pathway. First, there are precursors to the micoRNAs that are transcribed in the plant nucleus. They are processed out by endonucleases called dicers. There are four dicers in plants; small siRNA is introduced into a RISC complex (RNA-induced silencing complex) and the complex is aided by another by another group of genes called argonauts. In the RISC complex, the passenger strand is degraded and the guide strand is used by the RISC complex to guide that complex to a target which is a mRNA that interferes with the translation of that mRNA, or results in slicing of that

mRNA. Fungi do not have this microRNA but they do have another arm of the RNA silencing pathway that is involved with defense. In plants, there is a response to virus infection in the production of dsRNA by host RNA viruses and another set of dicers degrade this dsRNA or structured regions of the plus strand RNA into small RNAs and they are incorporated into RISC complexes and go back to the viral RNA and destroy it. That is the basis for the anti-viral defense mechanism. That can be amplified again involving other dicers and RNA-dependent-RNA polymerases.

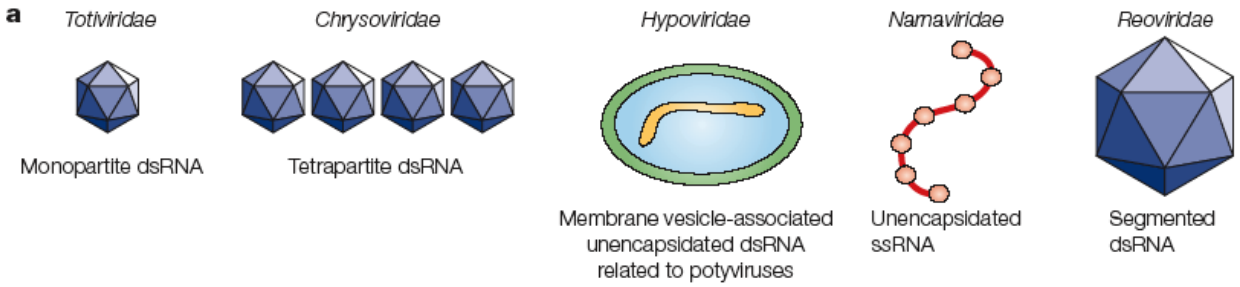
RNA Silencing in Plants

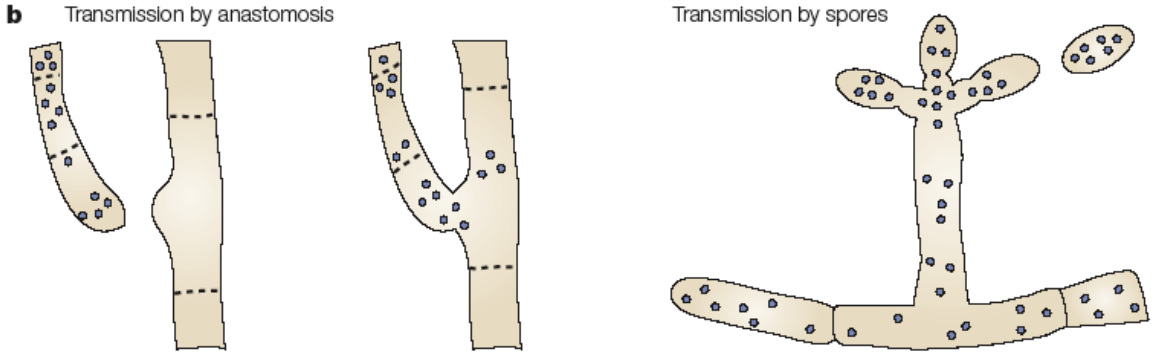


It is a little simpler in the best characterized fungal RNA-silencing pathway, *Neurospora*. There is a vegetative RNA-silencing pathway and another silencing pathway that occurs only during meiosis. The genes that are involved in quelling were identified in a number of different ways and then characterized. They include helicase that is involved in recognizing transgene RNA, transposable element RNA and in some way, results in the production of aberrant RNA that is recognized by RNA-dependent RNA polymerases in the cell to produce dsRNA which is a substrate for the dicer. There are two dicers that are somewhat redundant in that they both can be involved in the production on the small RNAs that are incorporated into the RISC complex. It hasn't been possible to ask whether this silencing pathway in *Nerospora* is important for anti-viral response because there is no good viral system in *Neurospora*. However, it sets the stage to ask similar questions in *Cryphonectria* where there is a good virus system.

They looked at dicers first. There are two dicers that are very similar to the dicers in *Neurospora*, Dicer-1 and Dicer-2. They knocked them both out and there is no phenotype that they can identify in the absence of virus infection.

There are five virus families that have been characterized that that replicate in *Cryphonectria*. They have very different replication mechanisms so they will be very useful in looking at a large number of basic questions on how these viruses interact in the RNA silencing pathway.





When they infected the WT and Dicer-1 mutant, they responded in the same way as the virus-infection, whereas with the Dicer-2 mutant and the double dicer (Δdcl-1/Δdcl-2), they were severely affected by virus infection, indicating that Dicer is somehow involved in antiviral defense response that prevents virus from causing a dramatic phenotypic change.

There are two argonautes in *Neurospora* and four argonaute genes in *C. parasitica*. Argonaute 2 and 4 are the orthologs of the two *Neurospora* argonauts. Qihong Sun (in Nuss' lab) has knocked out all four of the argonautes and again there is no obvious change in phenotype in the absence of virus but arogonaute 2 gives a very similar to the Dicer-2 knockout. That means that they have a system that has a lot of advantages for looking at RNA silencing as an antiviral defense mechanism. The advantages of the hypovirus/*C. parasitica* system for studying RNA silencing antiviral defense are:

1. Simplified pathway: single dicer and single argonaute.
2. Natural host to viruses with different replication strategies representing five different taxonomic families.
3. Facile genetic manipulation of hypovirus and host genomes.
4. Evolutionary position relative to animals and plants to ask about the evolutionary pathway of the RNA silencing pathway.

Nuss presented details of a recent observation that has broader implications than just the importance of the RNA silencing pathway for hypovirus replication and the effect of the virus on plant/host interaction.

This begins with the characterization of small RNAs that are generated by dicer on the viral RNA after infection. They produce small 20-21 nt virus-derived RNAs that can identified on gels or cloning. They turn out to be produced by Dicer-2. A number of the dsRNAs were cloned and 73% of the clones were derived from the virus. Where do these come from on the viral genome?About 60% come from the plus stand and 40% from the minus strand. When they looked to see how they were distributed along the genome, they noticed some areas where they did not find much, especially on the minus strand. A big hole was found around the area where polymerase is encoded. They thought the reason that there was a very low number of dsRNA in the hole was the propensity for these viruses to generate interfering RNAs. At one of the early NE-140 meetings, there was discussion about defective RNAs that were found in the population of dsRNAs extracted from the virus-infected strains. Patterns changed over time; there were large, medium-size and small RNAs. In characterizing the medium-size RNA, it was found that the 5' and 3' ends remained intact and the deletion occurred in the middle. They recently cloned and characterized the predominant m-species to see if the deletion occurred very close to where they saw the 'hole'.

- Viral RNA recombination is an important component of virus evolution that contributes to the emergence of new viruses and the generation of internally deleted mutant RNAs [Defective interfering (DI) RNAs] that are derived from, and dependent on, the parental viral genomic RNA.
- DI RNAs can suppress the parental RNA accumulation leading to attenuation of symptoms and persistent virus infections.
- Rapid viral RNA recombination also appears to be an underlying cause of the instability and deletion of foreign, non-viral sequences from recombinant viral RNA vectors.
- Genome instability presents one of the most important obstacles for use of recombinant RNA viruses as gene expression vectors for a variety of practical applications, including gene therapy.

They found that when they cloned the m-species, the region that corresponded to the polymerase was absent so it does correspond to the region that is very deficient in dsRNAs. More importantly, they observed that the Dicer-1 mutant in the WT strain produced these defective RNA at a very high rate. In the Dicer-2 mutant, only the full-length viral RNA was present. It looks like Dicer-2 is required for the production of interfering RNAs. In other research, they found that DCL2 is required for instability of foreign sequences in recombinant hypoviruses. Also, they found the first evidence for a role of RNA silencing in viral RNA recombination.

Hypovirus papain-like protease p48 is required for initiation but not for maintenance of virus RNA propagation in the chestnut blight fungus *Cryphonectria parasitica* In work conducted by Fuyou Deng on p48, it was found that:

1. p48 is required for initiation of CHV1-EP713 RNA infection in *C. parasitica*.
2. The Δp48 mutant virus can be rescued when p48 is provided *in trans*. Demonstrated by transfection, transformation and anastomosis.
3. p48 is not required for hypovirulence, but does contribute to viral RNA accumulation levels.
4. Surprisingly, the Δp48 mutant virus retains replication competence in the apparent absence of p48 following transmission to wild-type *C. parasitica* and successive sub-culturing.
5. Novel hit and run mechanism may involve p48-mediated conformation change, priming, or recruitment of other factors.

Transcription factors. In microarray studies looking at the effect of virus infection on transcript levels, there were 2,200 genes represented and three transcription factors identified as being responsible for virus infection. They took two of the three (identified by microarray), CpST12 which is related to a

yeast transcription factor required for mating and *pro1* which is the most recently documented down-regulated factor. They found the following:

Characteristics	*pro1* disruption mutant	*cpst12* disruption mutant
Vegetative Growth	Same as wild-type with increased aerial hyphae	Same as wild-type
Asexual Sporulation	No or few conidia produced under standard lab. conditions	Significantly more conidia produced than wild-type strain
Sexual Sporulation	Female sterile	Female sterile
Virulence	Virulent as wild-type	Significantly reduced virulence

It is remarkable that two of three transcription factor genes that were identified as being hypovirus-responsive (microarray analysis of 2,200 *C. parasitica* genes) turned out to be required for three major biological processes that are altered as a result of hypovirus infection. Genome sequence allows an examination of the influence of hypovirus infection on and the biological role of all C. *parasitica*-encoded transcription factors.

A lot of transcription factors have been mapped and identified. They have found:

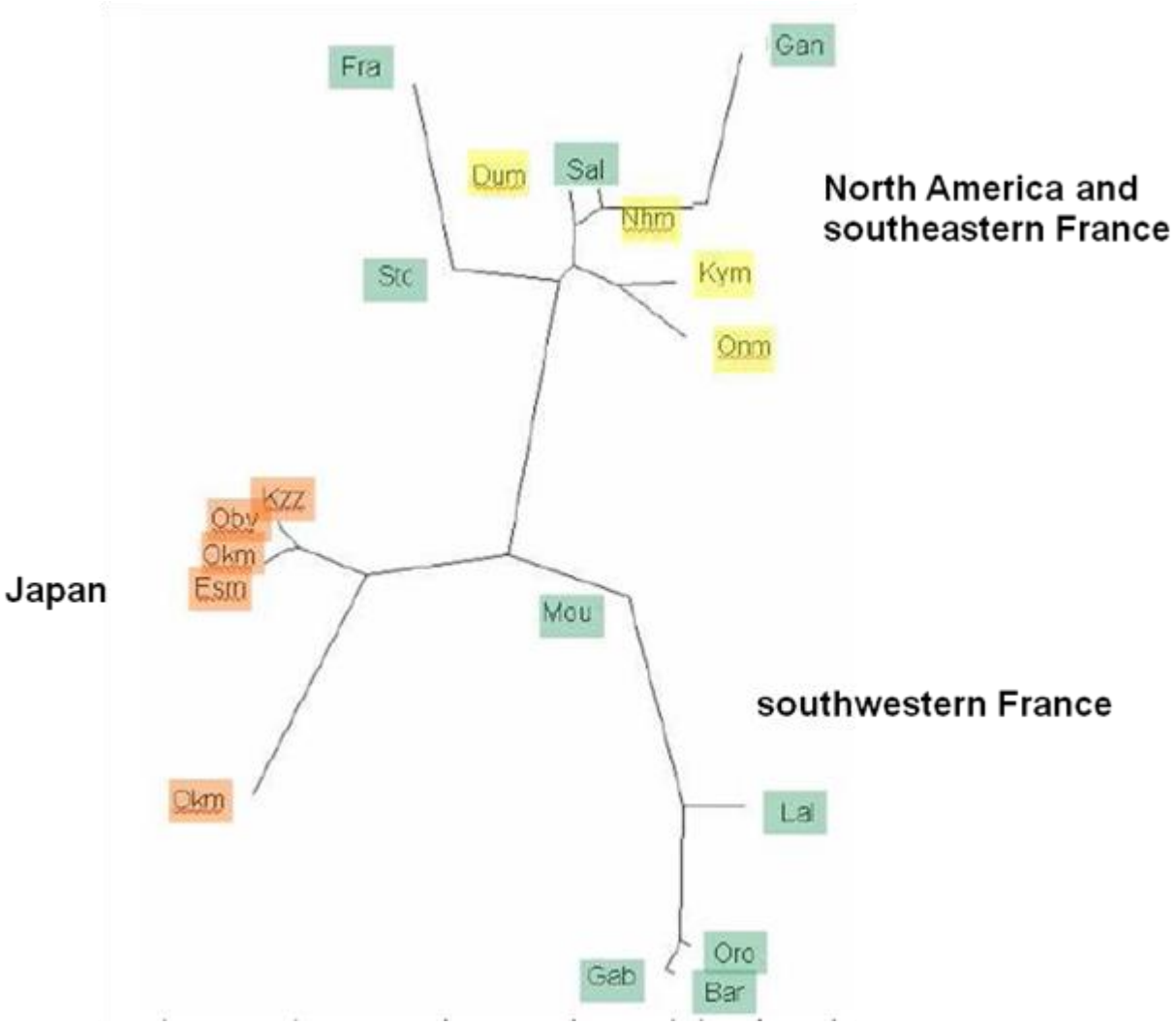
1. CPST12 is a member of C2/H2 zinc finger transcription factor family.
2. Over 20 members of this family found in *C. parasitica* genome.
3. PRO1 is a member of the Zn(II)2Cys6 fungal binuclear cluster transcription factor family.
4. Over 80 open reading frames with Zn(II)2Cys6 binuclear cluster motifs found in the *C. parasitica* genome.

Two manuscripts resulted from this work: (1) 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* Fuyou Deng, Todd D. Allen[#], and Donald L. Nuss Eukaryotic Cell (2007) 6:1286-1298; and, (2) Hypovirus-responsive transcription factor gene *pro1* of the chestnut blight fungus *Cryphonectria parasitica* is required for female fertility, asexual spore development and stable maintenance of hypovirus infection, Qihong Sun, Gil H. Choi and Donald L. Nuss, submitted to Eukaryotic Cell.

Michael Milgroom—Cornell University

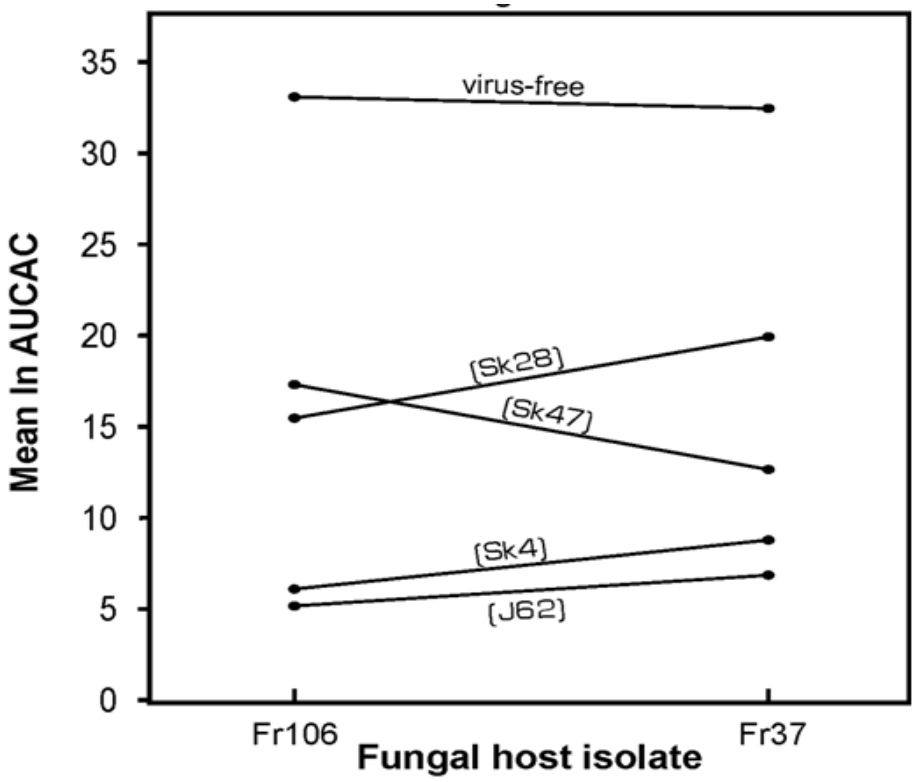
Milgroom limited his discussion to two topics: (1) Worldwide population genetic structure (with Cyril Dutech and Cécile Robin, INRA, Bordeaux, France; and, (2) biological control with hypovirulence in Macedonia (Kiril Sotirovski, University Ss. Kiril I Metodij).

Worldwide population genetic structure. Cyril Dutech, knowing that Milgroom had a *C. parasitica* collection from North America and Asia, wanted to put those isolates together with a collections from Europe for an intercontinental study. Dutech took 10 microsatellite markers and looked at 4 populations from N. America, 6 from Japan and 9 from southern France (most populations had 15 as a minimum sample size). Allelic richness (number of alleles found per locus) was greatest in Japan, followed by European and North America. He was surprised at the richness in southern France. There were three clusters as shown below:



Along the Pyrenees, there are many different vc types than are found in the remainder of Europe. He hypothesizes that there was a second introduction of *C. parasitica* into Europe. In southeastern France, there is very low vc diversity.

Biological control with hypovirulence in Macedonia. Milgroom visited a stand of trees in Genoa (northern Italy), where the dominant tree was chestnut. About 95-98% of the trees had cankers and most were very superficial. It was an eye-opening visit for him; as much as he has been a skeptic of hypovirulence, this stand of trees made him a believer in hypovirulence. This was in contrast to stands in southwest Macedonia near the border with Albania where there are lots of dead and dying chestnut trees. Sotirovski wanted to do a biocontrol study. They took two fungal isolates and introduce six viral isolates into each fungal isolate. Each fungal/viral combination was inoculated into 10 different healthy trees and they were followed over the course of five years. There was a lot of variation. They calculated the area under the canker-area curve (sum of area under each curve).



The important issue with the above graph is the action of Sk28 and Sk47 that are intermediate in size. Sk28 has a differential effect on isolate Fr106 than Fr37. The same was true for Sk47; there is specificity between viral strain and fungal host. All of the viral isolates (from Macedonia) shown fit into the Italian subgroup of CHV-1 hypoviruses.

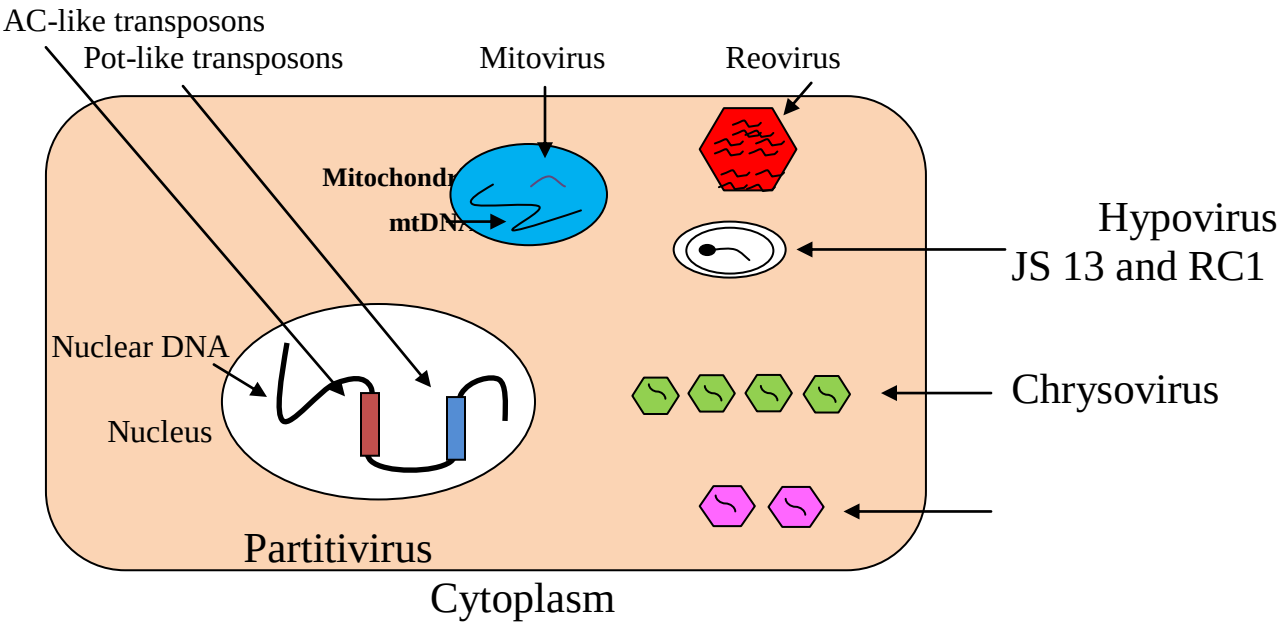
Bradley Hillman—Rutgers University

Hillman reported on the following:

1. Complete characterizations of two *C. parasitica* viruses and their genome sequences: RCI (Michigan); and, JS13 (Japan)
2. Genomic and proteomic analysis of interactions between *Lysobacter enzymogenes* and *C. parasitica* and between two reoviruses and *C. parasitica*
3. Examination of interactions between *C. parasitica* hypoviruses and reoviruses

The Ross Common isolate from Michigan (RC1) and a Japanese isolate, JS13, are both hypoviruses. Hillman showed a diagram of viruses that infect *C. parasitica*, shown below.

Cryphonectria viruses and transposons



Cryphonectria virus JS13 has a genome size of 8-9 kb and groups phylogenetically with two non-encapsidated viruses with lineages closer to dsRNA than ssRNA viruses. JS13 is related to *Phlebiopsis giantia* virus 2 and *Diplodia scrobiculata* virus 1.

Cryphonectria virus RC1 from Michigan has a genome size of ~4.4 kb (two segments, 2.8 + 1.6 kb); its phylogenetic grouping is unknown – continuation of work from Chris Smart, Cindy Paul and Dennis Fulbright. RC1 dsRNAs are likely cytoplasmic; dsRNAs 1 and 2 do not cross-hybridize. The total of ~2.6 kb of sequence done (1.6 kb from RNA1, 1.0 kb from RNA2) – no homology to known virus sequences have been identified to date; there is no putative RdRp sequence.

***Lysobacter* project**. *Lysobacter enzymogenes* (Gram negative, soil-inhabiting bacterium) is a fungal-infecting bacterium. Hillman reported on the genomic and proteomic analysis of interactions between *Cryphonectria parasitica* and *Lysobacter enzymogenes* and between *C. parasitica* and two reoviruses MyRV-1/9B21 and MyRV-2/C18. In an interaction, he is looking to see what genes are up or down regulated, from the bacterial side and from the fungal side. *Lysobacter enzymogenes* is a common bacterium and it has every secretion system known. A few of the secretion systems are poorly investigated. After a few hours of interaction between *Lysobacter enzymogenes* and *Magnaporthe oryzae*, bacterial attachment is seen polarly and then later, there is internalization of the bacteria. Finally, there is a lysis of cells. This is a bacterium that produces prolific amounts of enzymes and antibiotics. They want to look at how the bacterium gets into the fungal cell and allows the fungus not to go apoptotic. From the proteomic standpoint, they have some proteins examined from both the bacterial side and fungal side.

Reoviruses. He is following up on work done by Nobuhiro Suzuki (RIB, Okayama University). Reoviruses are interesting because the closest relative to *C. parasitica* reoviruses is a human pathogen, Colorado tick fever virus. The two reoviruses are both dsRNA viruses. They have 11 dsRNA segments in particles. The two viruses have different effects on the fungus and both reduce virulence dramatically. Viruses are inherited in conidia at a rate of 2-60% and there is 50% inheritance in ascospores. Both

viruses have been sequenced completely and there is less than 50% amino acid identity. Hillman was surprised at how unrelated they were given that they were isolated less than 20 miles apart from cankers in West Virginia. Hillman stated that the expression of CHV-1 p29, either as a transgene or from replicating virus, induces segment rearrangement in MyRV-1/Cp9B21. A lot more than two of the segments are rearranged. Expression of one protein causes, in the case of one segment, duplication of almost the entire open reading frame, and in other cases, the deletion of an entire open reading frame.

A summary of Hillman's collaboration with Suzuki is:

- Sun and Suzuki have shown that transgenic or natural viral expression of the multifunctional proteinase/silencing suppressor CHV-1 p29 results in reproducible mutation of dsRNA segments of Mycoreovirus-1/Cp9B21
- Hillman's lab is taking two approaches:
  - Examine the effects of CHV-1 p29 on a second reovirus, MyRV2/CpC18
  - Examine the effects of different hypovirus p29 homologues on reovirus replication

Jo Anne Crouch—Rutgers University

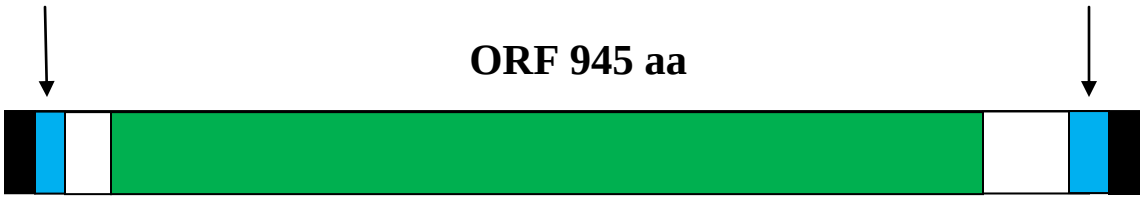
DNA transposons in the *Cryphonectria parasitica* EP155 genome. Crouch indicated that transposons are extrachromsomal elements that have the ability to jump around in the genome. Transposons code for a single gene, a transposase; they are bordered on both sides by direct repeats (see diagram below). They can significantly impact the way a host genome evolves. Transposable elements are important because they move about the host genome and insert into a host's DNA; thus, 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.

Crouch hypothesizes that there are four species of DNA transposons in *C. parasitica* strain EP155, *Crypt1, Crpyt2, Crpyt3 and Crpyt4*. *Crypt1* is part of the *haT* super family of transposons, while the other three species are from the *Tc1/mariner* (aka *fot1*-like) family DNA transposons. *Crypt1* was the first element identified in *C. parasitica* a few years ago. It is a Class II DNA transposon of the *hAT* family. *Crypt1* 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. ***Crypt1*** is seen below:

Inverted repeat 21 nt **Inversted repeat 21 nt**

CAGCGTTCCACACAAGTCAAG

CTTGACTTGTGTGGAACGCTG



Direct repeat 8 nt **Direct repeat 8 nt**

pMS5.1 CCCTCATG pMS5.1 CCCTCATG

Cosmid 3 GGTGGTGG Cosmid 3 GGTGGTGG

Cosmid 7 GTGGCCAT Cosmid 7 GTGGCCAT

There is very little identity between *Crypt1* and *Crypt2* as far as functional domains within the sequences. *Crypt1* is larger than *Crypt2* by about 400 amino acids. When a BLAST was done of these two against each other, there was amino acid identify in only 46-bp over two regions or 37%. Crouch was able to detect a total of 15 *Crypt1* copies across 9 scaffolds. Of those 15 copies, 10 were full-length and 9 have a continuous open reading frame; the other one is interrupted. About 60% have an open reading frame. Looking at the phylogeny, there is not a lot of variation within this population. It is actually a very small copy number for this class of DNA transposon. In contrast, *Fusarium* can number in the hundreds.

The evidence she used to show the four species is as follows:

Crypt2 was the second element identified. It is also a Class II DNA transposon but of the *Tc1/mariner* family. It was identified by the Hillman lab in early 2000's; data currently unpublished, and it is presumed active through presence in Dawe's *et al*. EST library. *Crypt2* is present in much higher copy number than *Crypt1*. With regard to *Crypt2* phylogeny, there are 56 full-length copies of *Fot1*-like transposons acrosss 18 scaffolds. In addition, there are 103 fragments that remain as remnants (63-1139 bp).

It is clear looking at the phylogenetic tree above that these three different subclasses are individually more closely related to sequences that are found in other fungal organisms. *Crypt2* is more closely related to elements in *Magnaporthe, Botrytis, Sclerotinia* and *Ophiostoma* than *Crypt3* or *Crypt4*. In looking at their structure, *Crypt3* is a little bit larger than *Crypt2* or *Crypt4*. There is also a difference in domains that are present. *Crypt2* has two DNA binding domains at the 5' end that are not present in *Crypt3*. In comparison of the actual sequence basis, there is not a whole lot of amino acid identity. In comparing *Crypt2* and *Crypt3*, there is amino acid identities in only 207-bp over four regions; this is only 37%. What is also distinctive is that there are 45 insertions on these *Crypt3* elements, relative to *Crypt2*. There is also very little amino acid identity between *Crypt2* and *Crypt4*; amino acid identities in 247-bp over two regions which equals 26%. *Crypt4* has 30 insertions relative to *Crypt2* and *Crypt3*.

With regard to the populations of *Crypt2*, *Crypt3* and *Crypt4* in the EP155 genome, *Crypt4* is likely an extinct species. There are only six copies present and all are interrupted by stop codons. No copies possess intact open reading frames. The 30 insertions are shared among all six copies, meaning that the progenitor also had to share those insertions. The same situation exists for *Crypt3*. There are seven copies, located on six scaffolds, and no copies have an intact open reading frame. There are 18-21 stop codons and the 45 insertions are identical in all members; thus, they must have entered with common ancestor.

Couch wondered if naming these elements using bioinformatics data is a valid approach. In her mind, these are distinct species. Hillman commented that retrotransposons fall into the virus nomenclature, while DNA transposons do not; they fall into gene catergories. This, therefore, puts DNA transposons into a 'no-man's land' between viruses and genes. Crouch commented that no one has done a comprehensive analysis of DNA transposons in filamentous fungi.

John Carlson, Pennsylvania State University

Molecular genetics. Genomic Tool Development for the *Fagaceae* (Funded by the National Science Foundation). This project is a collaboration with Ron Sederoff (PI), and Dahlia Nielsen and Chris Smith at NC State University, Bill Powell and student Kathleen Baier of the College of Environmental Science and Forestry at Syracuse, Paul Sisco and Fred Hebard at TACF, Jeff Tompkins and Meg Stanton at Clemson University, and Tom Kubisiak of the US Forest Service Gulfport, MS. The scientific advisory board includes Jennifer Koch, US Forest Service Delaware, Ohio, Jeanne Romero-Severson, Notre Dame University and Doug Cook, UC-Davis.

Goal: - Identify the genes in the Chinese chestnut genome for blight resistance.

Approach: - BAC libraries for Chinese chestnut
- Physical map of Chinese chestnut
- EST databases
- SNPs and SSRs from the EST database
- Genetic linkage maps for Chinese chestnut

Applications: - Tools for marker assisted selection,
- An integrated genetic and physical map, and
- Platform for targeted genome sequencing

During the first two years of the project, the group at Penn State completed the cDNA sequencing for the EST databases which were subsequently set up at Clemson. They generated approximately 380 Million bases of transcript sequences using the 454 pyrosequencing facility. The bulk of sequencing was conducted on Chinese chestnut and American chestnut cDNA libraries. They assembled 172 M bases of high quality Chinese chestnut cDNA sequence into app. 40,000 unigenes (32,738 transcript contigs and 7,301 singleton reads). For American chestnut, they obtained over 78 Million bases of cDNA sequence which assembled into 22,714 transcript contigs. They also generated over 66 Mb of cDNA sequence for northern red oak (*Quercus rubra*), approximately 48 Mb for white oak (*Quercus alba)*, and approximately 15 Mb for American Beech (*Fagus grandifolia*). All of the contigs were annotated using the BLASTx alignment tool, and the sequences of all reads, contigs and annotations will soon be available at the project web portal (www.fagaceae.org) hosted by the Clemson University Genomics Institute. A manuscript describing the project and all of the tools available at the project web portal is in preparation. One paper has already been submitted (in review at BMC Plant Biology) comparing the transcriptomes of Chinese chestnut and American chestnut cankers versus healthy stem tissues. Comparisons of the American and Chinese chestnut transcriptomes are as follows:

- The transcriptomes of American and Chinese chestnut are highly similar in gene function and diversity.
- The 454 cDNA sequences from chestnut cover all biological and molecular functions.
- Genes involved in response to biotic and abiotic stress/stimuli are perhaps more highly expressed in Chinese Chestnut than in American Chestnut

Genome sizes of some North American *Fagaceae* species

Species	Common name	DNA content (pg/2C)	Genome Size (Mbp / 1C)
Castanea dentata	**American chestnut**	**1.67 +/- 0.022**	**805 +/- 10**
Castanea mollissima	**Chinese chestnut**	**1.65 +/- 0.015**	**794 +/- 7**
Castanea sativa	**European chestnut**	**1.61 +/- 0.013**	**776 +/-12**
Castanea crenata	**Japanese chestnut**	**1.65 +/- 0.016**	**795 +/- 7**
Castanea seguinii	**Seguin chestnut**	**1.57 +/- 0.009**	**756 +/- 4**
Quercus alba	**White oak**	**1.59 +/- 0.07**	**766 +/- 13**
Quercus rubra	**Northern red oak**	**1.58 +/- 0.05**	**762 +/- 22**
Quercus velutina	**Black Oak**	**1.17 +/- 0.055**	**564+/- 27**
Quercus macrocarpa	**Burr Oak**	**1.34 +/- 0.03**	**646+/- 14**
Quercus falcata var pagodifolia	**Cherry Bark Oak**	**1.45 +/- 0.015**	**699+/- 22**

Quercus nuttalli	**Nuttall Oak**	**1.32 +/- 0.03**	**636+/- 16**
Quercus palustris	**Pin Oak**	**1.30 +/- 0.028**	**626+/- 13**
Quercus shummardii	**Shummard Oak**	**1.47 +/- 0.013**	**708 +/-6**
Quercus falcata var falcate	**Southern Red Oak**	**1.34 +/- 0.030**	**646 +/- 14**
Quercus nigra	**Water Oak**	**1.33 +/- 0.030**	**641 +/- 14**
Quercus phellos	**Willow Oak**	**1.35 +/- 0.041**	**650 +/- 20**
Fagus grandifolia	**American beech**	**1.13 +/- 0.014**	**544 +/- 7**
Fagus grandifolia	**American beech**	**2.03 +/- 0.008**	**978 +/- 4**

Linkage mapping studies. Masters student Kelly Deitrick conducted a small linkage mapping study with 17 RAPD markers that were previously identified by bulk segregant analysis (BSA) as differentiating pooled DNA from blight-resistant versus blight-sensitive trees. Eight of the markers were associated with blight sensitivity and 9 with resistance DNA pools. Deitrick conducted segregation analyses of these markers in the F_2 family in which the genetic linkage map and QTL were mapped previously by Kubisiak *et al*. This was done by PCR-RFLP to determine if the new DNA markers map to the same chromosome positions as the two major resistance loci. Primers also were made to two RFLP loci (145 and 175) from QTL on the original F_2 linkage map that were used by Laura Georgi at Clemson University to develop the first BAC contig. The online software MapManager was used to map the segregation data, with Paul Sisco's assistance and feedback. Deitrick was able to map six markers with the F_2 linkage population. In her final analysis, none of the new loci appeared to be closely linked to the QTL loci on the LG A and LG F linkage groups. However, the marker orders that she obtained differed from the original F_2 map. This may have been caused by use of a smaller data set, and a different mapping program.

Deitrick also conducted linkage analysis on a small F_1 population. The origin of this project was to compare the linkage maps of the F_1 and F_2 populations to provide insights into how the recently discovered reciprocal translocation(s) occurred. The F_1 population was obtained from 3 years of crosses of Mahogany pollen (from the CAES) onto burrs on the Alex-R American chestnut tree in the Black Moshannon state forest in central PA. All of the RAPD markers and SSR loci from the published F_2 map were screened with DNA from 40 individuals in the F_1 population, and those markers that successfully amplified were used for linkage analysis. The marker orders in the F_1 generation differed from the same the F_2 generations. This is what would be expected from reciprocal translocations, however her data set was too small for firm conclusions. The primary result from the F_2 and F_1 mapping exercises was training for Deitrick. The NSF *Fagaceae* genome project is now constructing complete F_1 and F_2 linkage maps with many new SSR and SNP markers which will result in much better maps and stronger comparisons.

Tom Kubisiak, USDA-Forest Service Southern Institute of Forest Genetics, Gulfport, MS

DNA marker (SSRs) development for various members of the *Fagaceae*. Kubisiak summarized the anticipated goals and presented the "why" behind using genetic marker mapping in Chinese chestnut. He also presented some of the work being done to help "finish" the *Cryphonectria parasitica* genome. A summary of the 454-based SSR marker development for the *Fagaceae* was as follows:

- 454-based DNA sequence data was obtained for expressed genes in various members of the *Fagaceae.* Species included Chinese chestnut, American chestnut, northern red oak, white oak, and American beech.

- The largest proportion of sequence data was collected for Chinese and American chestnut, followed by northern red oak and white oak and to a much lesser extent for American beech.
- Sequence data from all libraries were combined and these sequences were mined for in silico simple sequence repeats (SSRs).
- 454-based EST consensus sequences were searched for SSR motifs.
- Repeat regions had to have five or more 2 base repeats, four or more 3 base repeats, three or more 4, 5, 6 or 7 base repeats, or two or more 8 or 9 base repeats.
- The presence of multiple reads with different sizes of repeat was taken as evidence for a possible polymorphic SSR. In silico polymorphisms, either within a single species or between two or more species, were considered for primer design and further investigation.
- A total of ~570 putatively polymorphic SSRs were identified in ~460 different genes.
- 486 SSRs were chosen for primer design and screening.
- 486 SSR primer pairs were screened against a diverse panel of *Fagaceae:* two Chinese chestnut trees, two American chestnut trees, two European chestnut trees, two red oak trees, two white oak trees, two American beech trees, and four Chinese x Chinese cross progeny.
- Of the 486 primer pairs, 275 amplified single discrete loci but 211 failed to amplify discrete product.
- There are several possible reasons for failed primers: the consensus sequence was from all species combined; some primer pairs produced complex profiles and were hence counted as a failed pair; some gene contigs likely contained introns that resulted in products larger than the detection size limit of our DNA sequencer >600 bp; non-optimal PCR condition.
- Regardless, markers useful within as well as between various species were identified.

The following table briefly summarizes the results for the 275 markers that amplified discrete product within at least one of the species in the screening panel. (W/B indicates polymorphism either within or between genotypes of that species).

Species	Poly (w/b)	Mono	No Amp
Chinese chestnut	114	146	15
American chestnut	109	145	21
European chestnut	128	126	21
While oak	77	153	45
Red oak	57	107	111
American Beech	39	100	136

Genetic Mapping of Chinese Chestnut – DNA Extraction and Parentage Analysis

- DNAs were extracted from a total of 236 Vanuxem x Nanking cross progeny and 240 Mahogany x Nanking cross progeny (the common ramet was name Vanuxem).
- Enough DNA for both SSR and SNP analysis was obtained.
- A total of 16 SSR primer pairs developed from project 454-based sequence data were chosen for parentage analysis.
- Based on this data only one Vanuxem x Nanking progeny is definitively a pollen contaminant and only 5 of the Mahogany x Nanking are possibly pollen contaminants.
- Only five of the Vanuxem x Nanking progeny are dead and only four of the Mahogany x Nanking progeny are dead.

Why make a genetic map of Chinese chestnut?

- It is a main donor parent/species in interspecific breeding programs (harbors genes for resistance/tolerance to *Cryphonectria* as well as *Phytophthora*).
- Intraspecific crosses tend to have less problems when chromosomes pair and assort during meiosis.
- Have evidence for major chromosomal rearrangements between American chestnut and Chinese chestnut reciprocal tranlocations between at least one pair but maybe two pairs of chromosomes as well as a possible inversion. Thus, there may be major structural differences between American and Chinese chestnut.

Genome Sequence-Based SSRs for *Cryphonectria parasitica*. The goals of the SSR marker work were to:

- identify additional SSR markers in *Cryphonectria parasitica* using the full genome sequence
- design primers and identify polymorphic SSR markers near the ends of physical scaffolds
- use recombinational data to suggest genetic linkage between/among markers on different physical scaffolds to infer scaffolds that are likely to reside on the same chromosome
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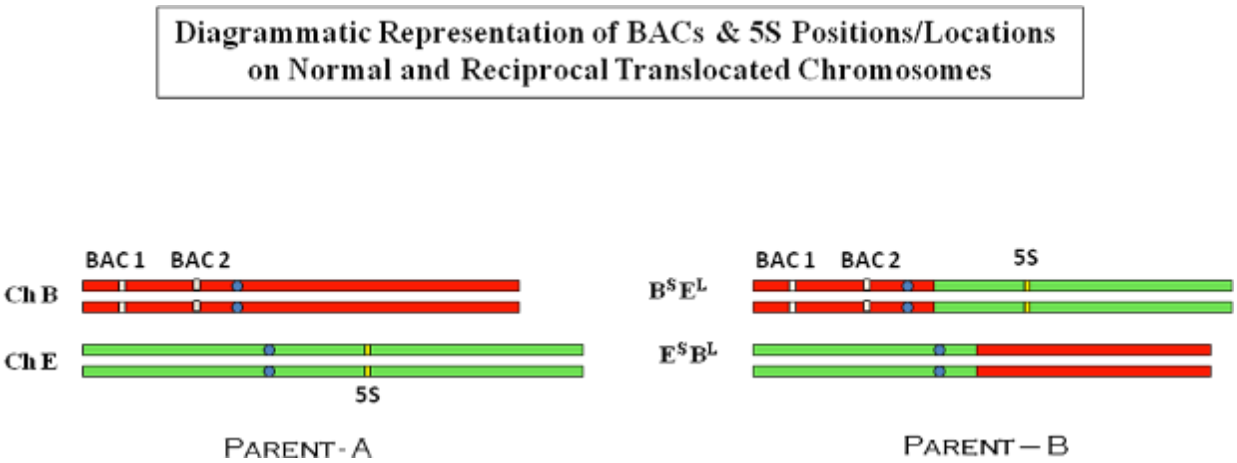
A total of 689 di- to deca-nucleotide SSRs were identified from the full genome sequence – 39 total scaffolds. Numbers of SSRs ranged from 118 on the largest scaffold to only one on some of the smaller scaffolds. A total of 141 primer pairs were designed from SSRs as close to the ends of scaffolds as possible Where previously mapped markers were found to be more terminal then no primer pairs were developed To increase our chances of identifying markers that might be polymorphic in our Cp mapping cross, when possible, multiple (as many as five, with one exception of six) SSRs per scaffold-end were targeted. All 141 primer pairs were screened on the parents (JA17 and X17.8) of the mapping cross to identify segregating markers Allele data for 96 the Cp mapping population were collected for 32 SSRs that were the most terminal markers in the genome scaffolds recombinational linkage data suggested that several scaffolds likely reside on the same chromosome: scaffolds 1 and 39; scaffolds 5, 12, 14; scaffolds 6 and 18; scaffolds 8 and 10; scaffolds 9 and 15; and, scaffolds 13 and 16.

Paul Sisco, The American Chestnut Foundation, Southern Appalachian Regional Science Coordinator

Cytological comparison of the chromosome structure of *Castanea dentata* and *Castanea mollissima.* Sisco reported on the work of Nurul Faridi (Texas A&M Southern Institute of Forest Genetics) and Laura Georgi (formerly at Clemson University). Ron Phillips (University of Minnesota and a former student of Charles Burnham) during the 2006 TACF science review suggested that there might be a reciprocal translocation because there are 11 linkage groups. Sisco showed slides that indicated reciprocal translocation between American and Chinese chestnut.

Laura Georgi has developed BACs from most of the linkage groups, and she has a good DNA marker for the two linkage groups that may be involved in the translocation. Faridi's hypothesis is found in the following diagrams. Perhaps in Chinese chestnut the 5S is on one chromosome and the two BACs are on the other chromosome (left diagram), where in American, the BACs and the 5S are on the same chromosome (right diagram). Within a species, pairing is fine, but in interspecific hybrids pairing is restricted, resulting in many duplications and deficiencies in resulting gametes.



The NSF group of scientists is aligning the physical map with the genetic map of Chinese chestnut cultivar 'Vanuxem'. Loci on both maps are in the same order but they are not the same distance. Loci on a genetic map may be very close but physically they may be very far apart because there is very little recombination. Likewise, there can be loci that are physically close but far apart on a genetic map because there is a lot of recombination.

Evidence for greater diversity of American chestnut in the South. With the help of grants from The American Chestnut Foundation, both Fenny Dane at Auburn University and Joey Shaw, Hill Craddock, and their student Meagan Binkley at the University of Tennessee at Chattanooga have been studying the genetic diversity of both American chestnut and Allegheny chinkapin in the South. It is clear from their studies that chestnut and chinkapin have interbred in the past, producing hybrid offspring. It is also clear that there is much more genetic diversity, at least in chloroplast type, in Allegheny chinkapins than there is in American chestnut. In terms of chloroplast DNA, American chestnut has two lineages, A and B. Lineage B is defined by a large deletion, found by Kubisiak when he did a survey of 900 American chestnut throughout the range. The deletion is more homogeneous in the northern part of the American chestnut range. There is a lot more chloroplast diversity in things that are phenotypically American in the south than there is from about northern West Virginia northward. In terms of chinkapin, it is much more diverse than American chestnut. Being a southern species, chinkapin was less impacted by the Ice Age.

Possible introgression of American chestnut and Allegheny chinkapin (Joey Shaw and Hill Craddock, University of Tennessee at Chattanooga). Shaw completed his Ph.D. at the University of Tennessee where he worked on *Prunus* species. Shaw found that among five *Prunus* species, there is one chloroplast genotype in the north and another genotype in the south. This suggests that chloroplast is more of a geographic indication more than a species indication. Shaw is continuing his work on chestnut and is preparing a publication on his findings.

Mark Double, West Virginia University

Introduction of hypoviruses at West Salem, Wisconsin (in cooperation with J. Cummings-Carlson, Wisconsin Department of Natural Resources; D.F. Fulbright and A.M. Jarosz, Michigan State University; and, M.G. Milgroom, Cornell University). The stand of American chestnut in West Salem became infected with chestnut blight in the late 1980s after 100 years of blight-free growth. Hypovirus introduction (individual canker treatment) was conducted from 1992-1997 (700 cankers on 133 trees received inoculum). From 1998-2002 hypovirus introduction was halted. Beginning in 2003 through 2008 following a steady increase in disease, hypoviruses were reintroduced. Two methods were

employed to evaluate whether biological control had been initiated: (1) punch treatment of individual cankers as done initially; and (2) wounding of trees by inoculating scratch wounds to the bark surface to create reservoirs of inoculum. In 2001 because of the increase in cankers in the stand, twelve permanent plots were established in three regions of the stand (Disease Center; Front and Beyond the Front). One-half of the plots in each region were punch treated and the remaining half were scratch-treated. Approximately 25% of the trees in each plot were untreated to assess tree-to-tree spread.

Hypovirus spread has been assessed 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.

In June 2008, a study examined the spread of hypoviruses to non-inoculated American chestnut trees outside the permanent plots to assess hypovirus movement beyond the treatment areas. Bark samples were removed from cankers on trees in a 15m zone beyond the perimeter of each plot. The bark samples were processed and the resulting colonies identified for presence or absence of hypovirus. Bark samples were taken from 460 cankers on 270 trees. Hypovirulent isolates were recovered from 82% of the trees on the perimeter of the 'Disease Center' plots (an area with the longest treatment history), 20% from of the trees outside the 'Front' plots; and 11% from perimeter trees in 'Beyond the Front' plots (the most recently infected portion of the stand). These data indicate that, with time, hypoviruses are spreading naturally at the site.

The biological control potential of *Cryphonectria parasitica* strains containing an infectious cDNA copy of the hypovirus CHV1-Euro7 (in cooperation with D.L. Nuss-University of Maryland Biotechnology Institute). This study 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 2008, there were 127 natural cankers in TG plots, 66 in CH plots, and 60 in V plots. Cankers were sampled, when detected and each November, to determine the hypovirus infection status of the thallus. Although the purpose of the spermatization treatment was to produce ascospores, many treated cankers also acquired hypovirus from the treatment inoculum and have begun to produce callus.

Some significant findings this past year include the increased detection of hypoviruses, both in the canker thallus and perithecial outcrosses. In 2007-08, hypoviruses were detected in cankers on trap trees, both in the thallus and ascospores. 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. Over eighteen-thousand individual ascospore colonies were examined from 829 individual perithecia in 2007. To date, 56,291 individual ascospores from 2,375 perithecia have been examined. Hypovirulent ascospore (HVA) isolates were collected from 79% of the spermatized cankers in TG plots. From those cankers, 52% of the perithecia yielded transgenic ascospores.

Tree mortality, as of July 2008, was greatest in the virulent plots (62%), followed by CH plots (40%) and TG plots (22%).

Rebecca Rush, an undergraduate student, examined virulent *C. parasitica* cankers from this study area for her SURE project (Summer Undergraduate Research Experience). Rush obtained one virulent isolate from each of the 36 cankers and those virulent isolates were paired with two the TG isolates (Ep155/pXHE7 and Ep146/pXHE7) and two CH isolates (Ep155/pE7TN and Ep146/pE7TN) used to introduce hypoviruses. Hypovirus transmission occurred at differential rates (25%, 3%, 6% and 0%, respectively for Ep155TG, Ep146TG, Ep155CH and Ep146CH). Twenty-three hypovirulent ascospore colonies, obtained from TG-treated cankers, were paired with virulent isolates from the 36 cankers. Hypovirus transmission rates of the 23 ascospore colonies differed from the two parental strains (Ep155/pXHE7 and Ep146/pXHE7) were paired. The capacity of the progeny to transmit hypovirus to the 36 canker isolates was highly variable, ranging from 4% to 52% transmission. The experiment demonstrated that hypovirus transmission is greatly influenced by the vegetative compatibility type of the donor strains. The increase in hypovirus dissemination that was evident when progeny of the transgenic strains were examined suggests that sexual reproduction between virulent and transgenic strains greatly enhances the potential for hypovirus transmission.

Backcross orchard for assessment of host resistance combined with hypovirulence (in cooperation with Fred Hebard and Bob Paris, 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. Seeds were replanted in May 2008. As of September 2008, 65% of the planted seedlings were growing. Drought in mid-summer 2008 may be responsible for some seedling death.

William MacDonald, West Virginia University

Infectivity of virulent and transgenic hypovirulent inocula of *Cryphonectria parasitica*, and the influence of inoculation methodology on canker development. (in conjunction with D.L. Nuss-University of Maryland Biotechnology Institute). This work was part of S. Kenaley's Ph.D. study. Deployment of transgenic (TG) hypovirulent strains of *Cryphonectria parasitica* may enhance the dissemination of hypovirus(es) and the establishment of hypovirulence in ecosystems with chestnut. However, in order to utilize these strains in biological control strategies, a more thorough understanding of the differences in the infectivity of TG and virulent (V) inoculum on canker development is necessary. The objectives of this study were to: (1) compare the infectivity of TG and V ascospores, conidia, and a mixture of mycelia and conidia; and, (2) examine the effect(s) of strain, inoculum type, wound size (diameter: 2.0 mm and 9.0 mm), and the delivery medium (potato dextrose and water agar) on the development of artificially initiated cankers. Strain, inoculum, wound size were the most significant factors contributing to infection. For all inoculation methods, those performed with V inocula resulted in the greatest percent infection (55.6%) and were significantly greater than inoculations utilizing TG inocula (24.3%). The infectivity of V inoculum types was not significantly affected by delivery method. Percent infection for V ascospore, conidia, and mycelia and conidia inoculum types was 47.9%, 58.3%, and 60.4%, respectively. In comparison, percent infection for the TG mixture of mycelia and conidia (41.7%) was significantly greater than TG ascospores (12.5%) and conidia (18.8%). For all treatment combinations, 9.0-mm wounds resulted in infection 66.1% of the time in contrast to 33.9% of the 2.0-mm

wounds. Inoculations with V conidia and a wound size of 9.0 mm resulted in the greatest number of infections (66.1%). Linear growth of cankers that resulted from infection was most significantly influenced by strain, delivery medium, and wound size. The mean linear growth of the V cankers (mean= 5.7 cm) was significantly greater than that of TG cankers (mean= 2.6 cm). The growth of cankers associated with 9.0-mm wounds was significantly greater than those initiated with 2.0-mm wounds, and wounds filled with potato dextrose agar resulted in larger cankers than those filled with water agar. Strain, delivery medium, and wound size were the most important effects between or among their associated interactions. Although this experiment did not attempt to examine the natural conditions for infection, it clearly identified the limitations of TG inoculum in initiating cankers and the subsequent growth that occurs. These limitations may affect the performance of TG strains in forest settings and thus influence their use in biological control.

Saprophytic Activity of *Cryphonectria parasitica* on American Chestnut Trees. Undergraduate, Miranda King, conducted a study to determine the potential of hypovirulent strains of *Cryphonectria parasitica* to grow saprophytically on chestnut stems and produce hypovirulent inoculum. This was accomplished by: (1) artificially inoculating American chestnut stems that ranged from healthy to dead with virulent and hypovirulent strains of *C. parasitica*; (2) measuring the growth and sporulation that result on these chestnut substrates; and, (3) sampling from the stems to ascertain the growth of the inoculated isolates.

The study was conducted at the Savage River State Forest near Grantsville, MD in a previously cutover area where sprout populations of chestnut were abundant. Trees were inoculated in June 2007 and included four different chestnut substrates for inoculation: (1) healthy trees; (2) dead trees that had been artificially girdled in December 2006; (3) healthy trees that were allowed to grow and sporulate for 8 weeks, then girdled (August 2007); and, (4) healthy trees that were allowed to grow and sporulate for 14 weeks, then girdled (September 2007). Thirteen trees were included in each of the four chestnut substrate categories for a total of 52 trees. The thirteen trees were subdivided into three groups, each consisting of four trees, including one control tree. Each group of trees was inoculated with a different inoculum, EP146 (virulent), EURO-7 (CHV1 hypovirus), or GrandHaven2 (CHV3 hypovirus). The thirteenth tree in each category represented a control that was inoculated with sterile water agar.

Two inoculation procedures were used, a circular punch wound and a 5 cm-wide scratch wound. Four cankers were initiated on each tree and all were inoculated with the same isolate type. Each tree had two punch wounds and two scratch wounds, 30 cm apart. When stems were girdled, the frill was made under the bottom canker. Once each wound was made, they were inoculated with one of the inoculum types. The inoculum mixture for each inoculum type was made by mixing 350 mL of 0.1% peptone water, 250 mL 2.5% water agar, and ten 10-day-old PDA cultures of either EP146, EURO-7, or Grand Haven2. Spore counts of each mixture were made to confirm that each inoculum type had approximately the same number of propagules. Results of growth were highly variable. For EP146-punch inoculated stems, the largest cankers were produced on living stems. The previously girdled trees produced larger cankers than the 8-week or 14-week girdled stems. Sporulation was greatest on previously girdled stems. For Ep146-scratch inoculated stems, the previously girdled stems produced the largest cankers. Again, sporulation was greatest on previously girdled stems. For EURO7-punch inoculated stems, the greatest amount of growth and sporulation were on previously girdled stems. The same pattern held for the EURO7-scratch inoculated stems. Grand Haven 2 growth was greater than the EP146 isolate, so that data was excluded.

The experiment indicated that there was no significant difference for growth of cankers when initiated on any of the four chestnut substrate treatments when inoculations were made in June, due to the

high amount of variability. Results suggest that linear growth is similar for the living, 8-week girdled, 14-week girdled, and previously girdled stems.

Alice Churchill, Cornell University

Characterization of a secondary metabolite pathway in *Cryphonectria parasitica* (in cooperation with Sandra Anagnostakis, CAES). *Cryphonectria parasitica* produces a family of orange and yellow aromatic polyketide pigments (anthraquinones) that color the mycelium and spores of the fungus when grown both *in vitro* and *in planta*. Production of this family of pigments is suppressed in strains of *C. parasitica* that contain European hypovirus. Therefore, this pathway has been of interest to us since it appears to be regulated, directly or indirectly, by hypovirus infection. Previously, we cloned, sequenced, and disrupted a *C. parasitica* polyketide synthase gene *PKS1* by *Agrobacterium tumefaciens*-mediated transformation (ATMT) and began to characterize several targeted gene knockout (KO) strains that resulted from these experiments. Early assays had indicated that *PKS1* is expressed in a medium supporting orange pigment production (PDB) but is not expressed in a medium that suppresses pigment production (SDY). These and other results suggested a correlation between orange pigment production and *PKS1* gene expression, which led us to hypothesize that *PKS1* encodes an anthraquinone synthase gene.

Churchill determined that three *PKS1* KO transformants generally have the same phenotype as wild type and ectopic integrant strains when grown on EP Complete Medium (CM), i.e., by visual examination of CM plate cultures, the levels of orange pigments produced by the KO strains were comparable to those produced by the control strains. She previously demonstrated no significant differences between the KO and control strains in colony growth rate on CM or in Golden Delicious apple fruits, suggesting no effect of *PKS1* gene disruption on the ability of the fungus to grow under either of these conditions. More recent analyses of *PKS1* gene expression in a variety of media demonstrated that expression is limited to growth of the fungus on PDB/PDA medium, whether orange pigment is produced or not in young or old cultures. Furthermore, the gene is not expressed after growth in SDY, YES, or EP Complete media, whether orange pigment is produced (EP Complete medium) or not (SDY, YES media).

Analyses of genome sequencing data confirmed that *PKS1* is a single copy gene. In combination with our gene disruption data and gene expression analyses, the results to date suggest that *PKS1* does not encode an anthraquinone synthase. A phenotypic difference between *PKS1* KO strains and control strains was detected only when cultures were grown in liquid 2X PDB medium. Under these conditions, control strains produce a brown pigment, whereas KO strains are cream/white. Quantitative analytical chemistry analyses are necessary to confirm the identity of the orange pigments produced by the KO strains in plate cultures and to identify the brown pigments from control strains that are absent in *PKS1* KO strains grown in 2X PDB liquid cultures. Virulence assays on dormant chestnut stems and *in vitro* sexual crosses conducted by the Anagnostakis laboratory demonstrated no measurable differences in virulence or perithecium formation in *PKS1* KO strains vs. control strains. Another notable observation was that multiple strains of *Agrobacterium tumefaciens* appear to have a nonspecific effect on virulence of *C. parasitica* transformants in chestnut stems and reduce aerial mycelium production *in vitro* in transformants created by ATMT. This effect has not been reported before for ATMT of other fungi.

Natural product extracts have been prepared from culture fluids and mycelia of control and *PKS1* KO strains grown in liquid 2X PDB in preparation for HPLC, HPLC-MS, and NMR analyses to identify the polyketide natural product(s) we predict should be missing in the KO strains. The chemical product of the PKS1 enzyme could be a known polyketide from *C. parasitica* and/or other fungi or a novel compound not previously reported in the literature. Manual annotation of all PKS genes in the *C.*

parasitica genome, as well as other key secondary metabolite genes (such as nonribosomal peptide synthetase genes) is in progress.

Angus Dawe—New Mexico State University

Identification of proteins altered in accumulation by hypovirus infection using 2D electrophoresis. Changes in protein production of hypovirus infected strains has been explored by separating total proteins on 17 cm isoelectric focusing strips (pH range 3-10) and then running these on large formatted SDS-PAGE gels. Following staining, the gel images were false colored and overlaid to identify differentially expressed proteins. Two clear spots present on one strain but absent from the other were excised from the gel and sent to the Proteomics facility at Baylor College of Medicine for analysis by mass spectrometry. In this manner, they identified important enzymes in glycolysis as being influenced by hypovirus infection. Two important enzymes (enolase and bis-phosphate independent 2-phosphoglycerate mutase) were identified. Enolase was absent from hypovirus infected mycelium, whereas bis-phosphate independent 2-phosphoglycerate mutase increased in hypovirus infected mycelium. Thus, glycolysis is affected by hypovirus infection. There are likely impacts on related metabolism (amino acid anabolic pathways lead off from pyruvate/PEP, downstream disruption of respiration). This finding was supported by microarray analysis. Todd Allen noted earlier genes in glycolysis being affected; Allen felt that there is also a clear effect on mitochondrial function.

In an effort to measure metabolic activity, equipment in a colleague's lab, Wayne Van Vorrhies, was used. A direct measurement by infra-red gas analysis of CO_2 and O_2 quantities in controlled samples of *C. parasitica* mycelium was conducted by placing small (0.5 cm^2) plugs of mycelium incubated 1 hr in a closed chamber. Quantitative CO_2 production and O_2 consumption was measured. Dawe found that the metabolic rates declined with the age of the colony. Also, metabolic pathways change with aging. Next steps are to validate 2D data by testing directly for enzyme activities and determine if this is transcriptional control.

A potential controlled expression system for *C. parasitica.* They would like to have a method to efficiently control ectopic gene expression (to express different genes from different organisms to look for complementation). They looked at a copper-mediated control system used in *S. cerevisiae.* There was a recent paper that identified a new element from *Cryptococcus* that showed promise (Ory et al., 2004). The premise is that a copper transporter, in the presence of copper, is not required and is therefore turned off by the transcription factor that physically binds copper ions. The presence of copper means much reduced or no transcription. If there is an absence of copper, there should be high expression (at least that is what was found in *Cryptococcus*). Dawe used EP155 with and without copper (on PDA) and saw no effect. *C. parasitica* transformants were grown in liquid medium in PDA plus either 200 μM BCS (a copper chelating compound) or 25 μM copper sulfate. EP155 grew on all media. Thus, *C. parasitica* does not seem to require copper. However, it does have a copy of the CTR4 gene, so it does have a copper transporter. In order to develop a new tool for gene analysis in *C. parasitica*, they cloned a region upstream of a putative copper transporter. Based on observations in the fungus *Cryptococcus neoformans*, this sequence controls transcription of the transporter gene in the presence (off) or absence (on) of copper ions. Using the *C. parasitica* genome information, they created a reported construct using GFP and assayed for the ability to control GFP production. Using these conditions, the production of GFP was reduced to near-zero when copper was in excess, but was highly expressed when copper was not available. Growth experiments on solid medium indicated that there was no significant change in phenotype of EP155 if copper was absent or present in excess. This promoter region, once validation is complete, will be a useful tool for controlled experiments in *C. parasitica*.

Rachel Acuña —New Mexico State University

Investigating BDM-1 Phosphorylation and consequences of Casein Kinase 2 (CK2) inhibition. BDM-1 is a phosphoprotein that is required for G-protein signaling in *C. parasitica* and is functionally associated with the Gβ subunit CPGB-1. In 2008. 24 site-directed mutants affecting the putative targets for phosphorylation by casein kinase 2 (CK2) were made, plus six additional mutants affecting possible N-glycosylation sites and sites for Protein Kinaase A activity. Analysis of the migration of the different mutants by electrophoresis has enabled the identification of three of the five consensus CK2 sites on BDM-1 as being phosphorylated. However, studies of the effects of these mutants on pigmentation, laccase production, colony growth and virulence indicate that the phosphosylation of BDM-1 is not required for these phenotypes. This suggests that in fungi, phosducin-like proteins may be regulated differently than in mammalian systems in which CK2-mediation phosphorylation plays an important role. Currently, the possible impact of these mutations on protein turnover rates is being investigated.

The activity of ellagic acid, a compound common in many plant tissues that is a natural and potent specific inhibitor of CK2, is being explored. Ellagic acid does inhibit the growth of *C. parasitica* when incorporated into PDA plates. However, application of inhibitory concentrations of ellagic acid on a daily basis to stems infected with EP155 showed no significant impact on canker formation.

Hilary Boyer, New Mexico State University

Cis-regulator regions controlling expression in *bdm-1* and *cpgb-1*. The Angus lab has designed a reporter system based on GFP to examine genomic regions required for the control of expression of these two genes. *cpgb-1* is a gene encoding the *C. parasitica* G-beta subunit 1, and *bdm-1* is a gene encoding the beta disruptor mimic factor 1. In 2007, Amanda Kemp reported on the beginning analysis with *bdm-1*. This has continued and they now have six reporter constructs and validated expression controlled by regions of 1726, 1243 and 818 bp upstream of *bdm-1*. Fragments representing 457, 279 and 144 bp are currently being analyzed. Interestingly, hypovirus infection appeared to reduce the GFP expression, but only for the larger two fragments. This effect disappeared for the 818 bp fragment, which was the same as uninfected mycelium. This suggests there could possibly be a hypovirus responsive element in the range of 1243 bp to 818 bp, although further studies are needed to confirm.

Boyer has generated five constructs to similarly analyze the putative promoter region of *cpgb-1*. She has identified one possible enhance region between 555 and 800 bp upstream of the start codon, and preliminary evidence indicates that at least (bp) are required for any transcript to be produced. Further analyses are ongoing for both genes.

Gloricelys Rivera, New Mexico State University

Identifying Circadian-Clock Genes in *C. parasitica.* Using the available genome sequence data for *C. parasitica*, she has identified putative components of the circadian rhythm pathway based on sequence conservation with known compounds from *Neurospora crassa*. Clock rhythms in N. crassa include: (1) *white-collar* genes (*wc-1 and wc-2*) - (photoreceptor) and in response to it, turns on *frq*; (2) frequency gene (*frq*) - central component of the clock; and, (3) *clock-controlled* genes (*ccgs*) - involved in conidial development. Other genes related to the circadian clock include: (1) Protein Kinase C (PKC) - regulator of light responsive genes and phosphorylate WC-1; (2) VIVID (VVD) - a WC-dependent light photoreceptor in *N. crassa*. Regulates light responses and entrainment of the clock; and, (3) NOP-1- *N. crassa*-opsin-1- a group of light-sensitive membrane receptors found in photoreceptor cells. Rivera's objective was to investigate if *C. parasitica* has a light detection/response components as *N. crassa*:

- identify light response genes

- provide evidence of the light-responsive nature
- detect and quantify the genes at different time points

For the light response genes, she searched the Broad Institute/MIT/Harvard database for *N. crassa* genes sequences and compared them to the
genome sequence database of *C. parasitica* using the BLASTN algorithm. Her results on circadian clock genes similarity is listed in the following table

Protein	% Identity
WC-1	59%
WC-2	57%
FRQ	52%
CCG-6	59%
VVD	56%
PKC	64%
NOP-1	63%

For evidence of light responsive nature, she designed a photography experiment in which shots were taken at different times of the day, using EP155 and EP155/CHV1-EP713

In summary, she has successfully identified putative orthologues of light response genes in *C. parasitica* showing an average identity of 59% indicating a conservation between species. Her results suggested a response to light stimulus during growth of *C. parasitica* on solid medium. Via RT-PCR, she was able to demonstrate transcription of the genes *wc-1*, *wc-2*, *frq*, *ccg-6*, *vvd*, *pkc* and *nop-1* in *C. parasitica*. Further studies, such as gene knockouts, are ongoing.

Susan Howard, Michigan State University

West Salem—what else is going on? For hypoviruses to work in a real biological setting, they must be able to move within the tree and within the forest. West Salem can answer some of these questions concerning hypovirus spread. Can we see this happening? What is happening outside the twelve permanent plots that were established in the West Salem chestnut stand in 2001? Howard showed slides of swollen cankers and asked if these cankers harbor hypovirulent strains. She then asked the question, 'if a canker on a main stem is treated with a hypovirulent slurry, do adjacent sprouts automatically acquire hypoviruses?' Howard indicated that all possible combinations of hypovirus/no hypovirus could be found. Howard chose trees, beyond the trees that Mark Double chose for an assessment of movement beyond the permanent plots. Howard sampled cankers using a bone marrow biopsy instrument and rated cankers using a 1-4 rating scale (1=good callus response and 4=no callus response). She found that 78% of the cankers exhibited callus (rating 1 or 2). Howard found the CHV1/Euro 7 hypovirus in four of the five plots she sampled. Twenty-two percent of the cankers contained the hypovirus. Of the cankers that harbored hypovirus, two-thirds were from cankers rated 1 or 2 while one-third of the cankers were rated 3 or 4. Her conclusions were as follows:

- If a trunk is treated, sprouts are not necessarily protected.
- Euro-7 hypovirus has spread even beyond expanded plots at West Salem.
- Swelling cankers don't necessarily harbor hypovirus.

Josh Springer, Michigan State University

Matrix demography. Matrix demography suggests that 1-10 cm trees will respond best to hypovirulence. To test this prediction, he will introduce a local hypovirus, Grand Haven 2 (GH2) into

three populations in Michigan. Last winter, Springer and Jarosz collected 246 canker samples from: (1) Leelanau, a stand which was infected in 1997; (2) Missaukee, a stand infected in 1998; and, (3) Stivers, a stand northwest of Cadillac infected prior to 1990. Springer is determining the vegetative compatibility types of cankers at the three sites and is in the process of introducing GH2 virus into each vc type. Cankers at the three sites will be re-inoculated with the resident strains containing GH2 in the spring of 2009. The progress and success of the hypovirus will be tracked for the next few years.

Trends in tree growth: a 12 year retrospective. Jarosz has conducted a census of six MI populations since 1996. The following stands have been censused:

- 2 diseased (Stivers and Missaukee diseased)
- 2 initially healthy (Leelanau and Missaukee healthy)
- 2 recovering (County Line and Frankfort)

  The following data has been collected:
  - Diameter
  - Disease rating
  - Number of stems
  - Production of seed

Springer showed graphs of trend of trees at the six sites. At the two diseased sites, Stivers first became infected before 1990; there were only 16 large trees (>10 cm) at that time; they all died by 2003. There is, however, a stable population of understory trees at Stivers. In Missaukee diseased, the stand began with 61 large trees in 1996 and most of those trees have died and resprouted, becoming understory trees. Many of the large trees are dying. In the two initially healthy populations, Leelanau first became infected in 1997 and Missaukee in 1998. In these types of populations, once disease comes into the populations, there is death and dieback. All of the decline in these two populations is attributed to chestnut blight. In the two recovering populations (County Line and Frankfort), there is much more stability than in any of the other population types. County Line is continuously stable over the census period. In the Frankfort site, there is a slight decline with minimal death; a changeover occurs from overstory to understory rather than to dead trees rather than large trees simply dying and not becoming understory as happens at the Leelanau site. In comparing the cross-sectional areas of overstory trees in the six populations, diseased populations experience large amounts of negative growth, initially healthy populations show slightly negative to slightly positive growth, and recovering populations show slightly to more positive growth per existing amount of cross-sectional area.

Springer's conclusions were:

- Growth (and survivorship) of recovering populations is similar to or better than initially healthy or diseased populations.
- Successful vertical and horizontal transmission of hypovirulence is key to ecological recovery.

Bill Powell and Charles Maynard—SUNY-ESF (submitted report)

Inoculating the 2006 and 2007 field tests. In 2006, they planted the first four transgenic American chestnuts in the field representing one transgenic event, LP-2V28, which was named "Wirsig". In the spring of 2007, 17 additional transgenic American chestnut trees were planted, 12 of which are in an experimental planting with an equal number of Chinese and American chestnut controls. This spring, the original Wirsig tree was inoculated as well as the most uniform 18 trees of the 2007 planting (6 transgenic, 6 Chinese, and 6 American seedlings). To date, the American chestnut controls have developed lethal cankers and the Chinese chestnut have formed superficial cankers. The Wirsig trees have lethal cankers but have significant swelling indicating a defense reaction greater than the wild-type American controls. The Wirsig trees have very low oxalate oxidase expression due to a promoter

deletion in the vector pVspB-OxO, therefore detecting a defense reaction at this low level of transgene expression is encouraging.

New binary vectors and new transgenic events. During 2006, Dr. Will Rottmann (ArborGen LLC) used two of our vectors, pVspB-OxO and pSE39, and one of ArborGen's vectors, pWVR31, to construct three new vectors, which they began to use in the 2007 transformation experiments. The first new vector, pTACF3, contains the oxalate oxidase gene driven by a repaired VspB promoter (replacing an ~700bp deletion in pVspB-OxO). The second vector, pTACF6, contains our ESF39 antimicrobial peptide gene driven by our chestnut ACS2 vascular promoter. This second vector has also been used by Dr. Scott Merckle in his transformation experiments. The third vector, pTACF7, contains both these gene constructs. All of the pTACF vectors contain the *nptII* gene, but none contain GFP. Therefore, co-transformations were performed using these vectors along with the control vector, pGFP, which contains a *bar* gene and a *gfp* gene to aid in selection.

They are currently using the following vectors in transformation experiments: pTACF3, pTACF6, and pTACF7 (described above); pCWEA1 (ESF12 and AcAMP1.2 antimicrobial peptide construct) and pCA1 (AcAMP1.2 antimicrobial peptide construct), both previously shown to enhance pathogen resistance in transgenic poplar; and p35S-CNO (chitinase – NIa proteinase - oxalate oxidase self-splicing construct). They are also in the process of constructing a vector to constitutively express oxalate oxidase to use as a control.

To date, they have produced three new transgenic events from the somatic embryo cell line WB275-27 using pTACF3. These three events, named AN-2XG1, AN-2XG4, and AN-2XG5, have been regenerated to the shoot stage. All three express the OxO gene to various levels and tissue specificity. All are currently in the process of being rooted and acclimated. AN-2XG4 has been acclimated and has demonstrated much higher vascular expression than any other event to date (Figure 3). Two transgenic control lines, AN-2G2 and AN-2G3, were also produced and contain only the pGFP vector. The presence of transgenes has been confirmed by PCR in all these events.

Additional transgenic events are still in the somatic embryo stage, are GFP positive, and are being propagated for DNA extraction and PCR testing for their second vector. New transformation experiments are continuing.

Next generation of resistance enhancing transgenes. They are collaborating on the NSF funded, multi-institutional, *Fagaceae* genomics project from which several differentially expressed genes have been identified in resistant Chinese chestnut cankers compared to susceptible American chestnut cankers. One of these Chinese alleles, a putative diphenol oxidase/laccase gene, has yielded enough interesting data to prompt them to test it in an American chestnut transformation experiment. Therefore, they are in the process of cloning the full-length cDNA of this gene to be put in the next generation of binary vectors. A manuscript for publication describing this gene is being prepared.

New Growth Chamber Optimization Experiments and General Shake-Down. The American Chestnut Foundation helped them purchase two Conviron Model ATC-60 plant growth chambers. Each chamber has sufficient space to grow more than 750 plantlets, increasing their capacity more than ten-fold. With the old growth room, they were limited to controlling temperature and day length and (to a very limited extent) relative humidity. The new chambers allow for the control of those factors with far more precision, but in addition, light intensity and CO2 concentration can be controlled. When an environmental parameter is neither controllable nor measurable it is not terribly important to know the optimum for that parameter. However, with the new chambers, it becomes necessary to specify an acceptable range. They have, therefore, spent much of the last nine months optimizing environmental parameters. Because chestnut plantlets are difficult to produce in large numbers, we have also used seedlings. The following variables have been examined:

-CO_2: For several months they have been running one of the chambers at an elevated CO_2 level of ~1200 ppm. (Ambient CO_2 in the control chamber is approximately 500 to 700 ppm). They split several batches of plantlets between the two chambers for acclimatization. These tests are still in progress. Very preliminary observations indicate a positive response to added CO_2, but additional batches will have to be tested before they can publish or would be willing to adopt elevated CO_2 as part of the standard acclimatization protocol. A seedling study of different container types has been carried out, which was replicated in both chambers and the greenhouse. The + CO_2 chamber seedlings have grown substantially thicker stems.

-Container type: There is a plethora of different commercially available containers for growing tree seedlings. Stuewe & Sons, Inc., a nursery container supply company in Corvallis, Oregon, carries a large inventory from more than a dozen different manufacturers and was willing to supply small numbers of a wide variety of containers. Approximately a dozen of the most promising were evaluated, using seeds. The best containers for growing seedlings were the larger (10") Deepot¨ tubes or one of several square 6 to 8" Mini-Treepots¨. Because plantlets are much slower growing than seedlings for the first year, it may be optimum to grow them initially in smaller containers and transplant to a larger container after the plantlets begin to grow vigorously. As of August 2008, the 5" D19L container was chosen which holds approximately 350 ml of potting mix.

-Potting mix & Fertilization. Data from third round of fertilizer trials on chestnut seedlings is being collected and analyzed, but the tentative conclusion is that the best approach to growing chestnuts is to use Faford's Extra Fine Germination Mix and then fertilize at each watering with Peter's 20-20-20 + micronutrients at a rate of 100ppm available nitrogen.

-Nutgrafting. In 2006, Linda McGuigan grafted American chestnut tissue culture shoots onto American chestnut nuts following protocols from Ed Greenwell (http://www.accf-online.org/chestnut/nutgrafting.htm) and Joe Schibig (http://www2.volstate.edu/jschibig/nutgraftingbasicshandouts.pdf). Out of approximately 60 nutgrafts, three survived and were successfully planted in the field (scions WB 275-27 LP 28, WB 275-27 LP44 and a Pond 2 which was originally taken from the field). In 2007, Andy Newhouse repeated the procedure. Two of his nutgrafts survived and were planted in the field (scions WB 275-27 non-transformed and P1-1 LP38). On the basis of these pilot studies, they obtained a small grant from the Northern Nut Grower's association and hired an undergraduate student, Jennifer Lillie, to optimize the procedure. After numerous attempts to repeat the pilot study results without success for tissue culture shoots, she turned to larger scion shoots collected from field-grown trees. She was able to nutgraft these larger and well-lignified scions from the Wirsig tree. Six of these nutgrafts were planted in the field June of 2008 and six more are to be planted in the fall of 2008. She was also successful in a different grafting procedure in which the nuts were allowed to germinate, the top of the germinated shoot was cut off and the tissue culture shoot was grafted to the remaining stem. Six grafts are in the greenhouse (4 transgenic Pond 1-1 LP38 and 2 non-transgenic 30015-2) and will be planted this fall.

Fenny Dane, Auburn University (submitted report)

Chloroplast and nuclear DNA sequence information revealed 2 lineages within the American chestnut. One lineage is closely related to Allegheny chinkapin (*C. pumila* var. *pumila*) populations from the southern Appalachian mountain region. The other American chestnut lineage expanded in northerly direction following the Last Glacial Maximum and was accompanied by a gradual loss of diversity. Allegheny chinkapins and American chestnuts probably found refuge in similar habitats during glaciation periods. Hybridization between the species was evidenced by chloroplast haplotype sharing. This must have influenced the diversity of American *Castanea* species.

Business Meeting

Paul Sisco was elected Chair-elect. Bradley Hillman will chair the 2009 meeting, and agreed to host the meeting at the New Jersey shore in late September/early October to take advantage of cheaper rates. Hillman commented on membership in the regional project. Simply because researchers were members of NE-1015 does not mean that they are automatically members of NE-1033. Individuals must become official members—that entails filling out Appendix E on the NIMSS website (contact Rubie Mize [rgmize@AESOP.Rutgers.edu] if you experience any problems). As of October 1, 2008, the project officially became NE-1033. Hillman reminded the chair that the NE-1015 termination report is due in 60 days following the conclusion of the meeting.

Fulbright thanked Bill MacDonald and Mark Double for their work on the NE-1033 extension (2009-2013).

There was discussion about the next international chestnut conference. The International Society for Horticultural Sciences (ISHS) sanctions international meetings. At the IV International Chestnut Conference held in China in September 2008, the consensus of the conference attendees was that the V International Meeting, to be held in 2012, should be held in the U.S. When provided with options for a meeting site in the U.S., most of the attendees chose the Washington, DC metro area. Discussion centered around the National Conservation Training Center located in Shepherdstown, WV as a possible site. This site is close to Dulles airport and the ThorpeWood, Sugarloaf chestnut plantings. William MacDonald and Donald Nuss agreed to work together on the meeting arrangements.

Following the meeting, Fulbright and Jarosz provided a tour of two chestnut sites (Stivers and County Line).

Respectfully submitted,
Mark Double
West Virginia University
November 2008