NE-140 Technical Committee Meeting
Biological Improvement of Chestnut (*Castanea* spp.) and Management of Pests

Kellogg Biological Station, Kalamazoo, Michigan
October 22-23, 1999

Attendance:

California:	Pam Kazmierczak
Connecticut:	Sandra Anagnostakis, Phillip Gordon, John Anderson
Maryland:	Don Nuss, Lynn Geletka
Massachusetts:	Timothy McKechnie
Michigan:	Dennis Fulbright, Andy Jarosz, Anita Davelos, Dipnath Baidyaroy
New Jersey:	Bradley Hillman
New York:	Michael Milgroom, William Powell
North Carolina:	Robert Doudrick
Tennessee:	Hill Craddock
Virginia:	Paul Sisco
West Virginia:	William MacDonald, Mark Double, William Jones
Ontario, Canada:	Colin and Beatrice McKeen

A reception was hosted at the Kellogg Biological Station on October 21, 1999 from 7:30-10:30 pm. The meeting was called to order at 9:00 am on October 22, 1999 by Chairman Fulbright. He welcomed everyone and then introduced John Anderson, the Administrative Advisor for NE-140.

John Anderson, Director, Connecticut Agricultural Experiment Station

Anderson noted that project was renewed on October 1, 1998 and this was the first meeting since the project renewal. He reported that there was no USDA-CSREES representative in attendance, due in part to the Federal government's attempt to reduce expenses and limit travel. His main concern was naming the individual that will complete the meeting minutes within three weeks. In addition to the meeting minutes, the annual report is due March 15, 2000. It was noted that Hill Craddock agreed to write the meeting minutes and, as current chairman, Dennis Fulbright will write the annual report.

He concluded his remarks by stating that he hoped the USDA award will not diminish our productivity. He hopes the group will continue its excellent work and as a result, we will see chestnuts growing again in our forests and orchards.

<u>Objective 1.</u> To improve chestnut trees for timber and nut production, and determine the cultural requirements of chestnut seedlings in nursery and natural settings.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

Fat content in chestnuts. She discussed the nutritional value of chestnuts and showed a table of the food value of various chestnut species.

	Percentages			
Species	Fiber	Protein	Fat	Carbohydrates
Chinese	14	8	2	65
European	14	6	4	66
Japanese	14	8	0.4	90
American	19	10	10	40

She noted that most flavor of chestnuts is associated with the fat content. She discussed fat content in relation to various cultivars and open pollinated trees. The Connecticut Agricultural Experiment Station is the international registrar for chestnut cultivars. The cultivar list will appear on the CAES web site soon. There are approximately 2,000 named cultivars. This list will identify the origin of the cultivars; many have their origin in Connecticut with Arthur Graves and Richard Jaynes. The list does not include any European cultivars.

Open pollinated nuts are comparable to cultivars, except in fat content. She showed the results of three crosses; the results indicate that fat content is contributed by the pollen parent. Fats in chestnut are unsaturated fatty acids: palmitic; strearic; oleic; linoleic; and, linolenic. Fat content is very similar among chestnut species, except oleic acid content is very high in American chestnut.

Fat content in chestnuts is analyzed by a commercial laboratory; each test is $95. Tests are conducted with 30 nuts per cultivar. Fat content will be useful information for orchardists; they may want to use good pollinators to increase fat content in open pollinated orchards. There is no data to show how much environmental influence exists with regard to fat content. She did indicate that the pollen parent does not affect nut size.

Bauer Park. She reported on a 64-acre tract of land near the Long Island Sound; the land is located in Madison, CT. In order to study the correlation between water availability and rainfall, three rows of chestnuts were planted with 21 trees per row. Chinese, Japanese and American chestnut were all planted in May, 1999. Seedlings were protected with tree tubes. Some trees were lost to the 1999 drought; rows were replanted in August with seedlings.

Phillip Gordon, New York Botanical Garden

Root Crown Collar. He has spent the past 12 years looking at American chestnut. He talked about the root crown collar, found 1-3" below the soil line. This collar is a survival mechanism and it is found only in American chestnut. He read an excerpt from Hugh Gordon's book, *Principles of Gardening*, regarding the root crown collar. There is an asexual, vegetative means of propagation that goes on all the time when the tree top dies. He described it as embryongenesis, *in vivo*. Somatic cells go on to make a whole new plant. Shoots will grow for one year and then send down their own roots, separate from the parental root. This new shoot behaves just like a seedling. These trees can be genetically different from parent trees. Somatic variation is occurring from the epidermal cells of the tree. Arthur Graves was the first to show this phenomenon. Sprouts recycle outward from the parent tree. In this way, chestnut trees are immortal. Trees die, resprout and die again. This cycle tends to move new trees further from the original parent.

Gordon had the opportunity to visit two farms in which irradiated nuts from Al Dietz had been planted. While similar irradiated nuts were planted on both farms, the chestnuts were managed in very different manners. Both orchards had chestnut blight. Virgil Downs collected three generations of nuts and planted them; all were open pollinated. As trees died, he replaced them with seedlings that resulted from nuts. Harvey Lyle planted 80 trees thirty years ago; he did not replace trees as they died. He propagated generations of chestnut asexually. Thus, one orchard was maintained sexually (Downs) and the other asexually (Lyle). At the Lyle farm, there was prodigious regeneration; this occurred because of the asexual propagation brought about by the root crown collar.

Riparian Project. He reported on planting American chestnut along river banks. Gordon asked the question, 'What can American chestnut do for us?' The roots of American chestnut put down a tremendous root system. A single root system from a rye plant can produce 7,000 miles of root tissue. Woody plants are much more permanent. American chestnut seems to grow well along good clean water. In Connecticut, areas with good water contain both chestnut and maple species. With little flowing water, only maple can be found. The Bauer Park project involves school students. Chestnuts are collected each fall and planted in plastic jugs; seedlings are then outplanted along the Connecticut

River The trees stabilize the bank, utilize pesticide runoff; in short, they provide biological remediation. This process educates school students in all aspects of chestnut; they gain a thorough understanding of the tree, from blight to hypovirulence to utilization.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

Isozymes and Graft Unions. She is working with Greg Miller, a chestnut grower in Ohio. Miller grafts large numbers of cultivars and he encounters a tremendous amount of incompatibility in graft unions. She noted that Frank Santomour previously reported that incompatibility at graft unions was due to peroxidase isozymes.

Peroxidases are responsible for lignin formation and wound healing. There are two major peroxidases in chestnut, A and B. American chestnut has only band, A, and Japanese chestnut contains only band, B. Chinese chestnut can contain either A or B or both bands.

Santamour's data was shown:

Chestnut Graft Data, Success of Graft Unions

Length of Time	Same Isozyme	Different Isozymes
1 Year	35/45	35/71
4 Years	16/16	0/15

Miller uses chip budding. He has taken tissue from both the stock and scion and his data is similar to that of Santamour, especially in short-term survival. Miller is continuing this study to look at long-term survival (after three years). Santamour speculated that graft failure, due to isozyme incompatibility, occurs because there is a lack of lignin formation at the union.

Hill Craddock, University of Tennessee at Chattanooga

He reiterated the findings of Anagnostakis as he noted there has been incompatibility at graft unions in European chestnut for 2,000 years. The Europeans have compensated for graft incompatibility by grafting to mother trees.

TACF report. As the science cabinet chair of The American Chestnut Foundation, he reported that the backcross breeding program had its first review. The review committee, comprised of Shawn Mehlenbacher (Oregon State University), Ronald Phillips (University of Minnesota) and J.P. van Buijtenen (Texas A&M University), made 15 recommendations.

The American Chestnut Foundation would like to sponsor a symposium of species regeneration to be held in Chattanooga in April, 2000. Policy problems and problems with other taxa will be topics for sessions at the symposium. He requested suggestions for invited speakers.

Breeding work. In his chestnut breeding work, Craddock is using hybrid pollen from TACF's Meadowview farm. He also has some pure Chinese chestnut. He put out 400 bags this year but collected only 100 chestnuts due to the heat and drought of 1999. Trees in another orchard are dying from some unknown cause, possibly ink disease.

Chestnuts along Appalachian Trail. A student at UT-Chattanooga hiked the Appalachian Trail this summer and recorded the number of chestnut on the trail. He counted 40,000 chestnuts from Georgia to Maine and noted distributional patterns; many chestnuts were found in NC. The farther north he went, the fewer chestnuts he saw. The student did not encounter any chestnuts north of Massachusetts. This may be due to the elevation of the trail in New England. Twig samples were collected for the chestnut diversity study, conducted by Tom Kubisiak (see report from Robert Doudrick, page 15).

Paul Sisco, The American Chestnut Foundation

Science review. He noted the science review, as per Craddock. From Sisco's perspective, TACF's program is on-track. Sisco and Hebard concur with the review team's suggestions to put efforts

into additional sources of resistance rather than go on to the B_6. The first nut release is planned for 2006 to 2007.

Farm report. Trees continue to grow well. The *Pythium* problem encountered last year was not a problem in 1999, possibly due to the application of Ridomil into planting cylinders. Nut harvest was also good. TACF's Pennsylvania chapter has equaled the Meadowview's nut harvest, as that chapter harvested 3,000 BC_3 nuts in 1999.

Blight resistance from American parents. In one of their Clapper BC_2 progenies, the average resistance is much closer to that of Chinese that the typical Clapper BC_2. It is possible that the resistance contributed by the American parent may have been high. Length and width measurements of artificially established cankers are as follows:

American seedlings	11.3 cm
F_1	9 cm
Chinese seedlings	6 cm
Nanking cultivar	4 cm

Also in this test were full-sibling families (BB_2 x Clapper, CC_1 x Clapper, BB_3 x Clapper, and AC_1 x Graves). The average length and width measurements of these families fell in between the F_1 and the pure American seedlings. The American parent in the crosses was from Virginia; unfortunately this tree is now dead so no further tests can be run. The conclusion was not all American chestnuts are equally susceptible; some may contribute to the blight resistance in the backcross program.

'Mahogany' RAPD and RFLP markers. Most traits of chestnut (stem hairs, twig hairs, etc). are on linkage group C. There are 2-3 major loci that confer resistance. There seems to be a relationship between resistance and Chinese markers, using single-point trait correlations with molecular markers.

Eastern Hardwood Genome Initiative. One tree species, pine, has received a $4 million appropriation from Congress. Sisco would like scientists who are interested in hardwoods to come together as a unified group to sell this eastern hardwood genome initiative to Congress as one voice. There are a number of members of Congress that are interested in hardwoods; one member is Congressman Steny Hoyer, Democrat from Maryland. Thus, there may be some enthusiasm within Congress to support a hardwood initiative. Sisco feels that now is the time to approach Congress for money.

<u>Objective 2.</u> To better understand the interactions and ecology of this host/ pathogen/parasite system at the molecular, organismal and environmental levels to develop effective biological controls for chestnut blight.

Bill Powell, State University of New York, Environmental Science and Forestry

Transgenic American chestnut. The goal of their research is to produce blight resistant American chestnut trees using genetic engineering. He noted the areas of research between his lab and that of Chuck Maynard.

Powell's Lab:	Transgenic development
	Resistance testing of transgenics
	Poplar transformation
Maynard's Lab:	American chestnut transformation
	Tissue culture and regeneration

The various resistance genes are developed in Powell's lab and then transformed into poplar first. Tranfsorming genes in poplar takes 5 months compared to 2 years in chestnut.

They have designed nearly 50 anitmicrobial peptides and examined them using computer models. Fifteen of these designs were synthesized and tested *in vitro*. Three peptide designs that demonstrated high levels of inhibition to *C. parasitica* growth were chosen for further work.

Antimicrobial peptides pCWEA1

win3.12, a wound-inducible promoter

ESF12, an antimicrobial peptide (from frog)

Whole poplar and chestnut callus tissues.

Two other constructs, pCA1 And pC5A1, drive a peptide from South American grain.

Septoria/Poplar leaf disk assay. The third leaf down on poplar trees was used in the leaf disk assay. The leaf disks were placed on agar and then inoculated with the fungus. The plants with the transformant, ogy/CWEA1 had much less fungal growth.

Tissue culture. They do not have whole chestnut plants yet, but they do have callus tissue. Dr. Jun Wang has developed an assay for callus tissue. They are testing various proteins that will retard fungal growth. In order to assay the callus, a slow-growing isolate of *C. parasitica*, COL2 is inoculated onto the callus tissue. The rate of fungal growth is measured on a 5-point scale. The average of 6-7 calli are used; best results are after 8-9 days of growth. There is a level of resistance based on fungal growth.

Oxalate oxidase gene. Oxidase converts oxalic acid into H_2O_2 and CO_2. Leaf disks were used as an assay to test for this gene. Disks were soaked for 3 hours in oxalic acid (100 mM). In poplar, there appears to be some protection of cells with this enzyme. They hope to conduct further experiments with chestnut callus tissue to see if the gene is expressed.

Gene pyrimiding. They are using multiple genes.

Trichoderma endochitinase

Oxalate oxidase gene

Possible ESF protein

They want the genes to be very tightly linked. Hongy Gao has cloned this gene using a win6.39 wound promoter. The gene is being tested in a gene gun (transient genes) to see if the gene will enter leaf tissue and be expressed temporarily.

Stem-specific promoter. They are isolating a stem-specific promoter from chestnut. A cDNA library is being constructed and they hope to date 8 unique clones. The next step is to determine expression levels in other tissues, such as root, seed and pollen.

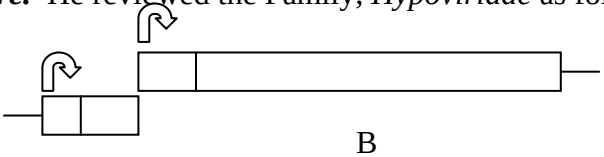
Their goal is to produce a transgenic American chestnut that contains 2-3 pathogen resistance genes. These genes will be tightly linked to prevent segregation and they will be under the control of a single promoter.

Somatic embryogenesis. There is a 2-week window each summer that seeds can be collected for somatic embryogenesis. Only 0.1% of the tissue collected become embryos. Once formed, however, they can be maintained for a long period of time. Once shoots develop, then roots can be promoted. Some of the embryos that developed into plants have been outplanted for three years. Seventeen months after beginning with a somatic embryo, they have a 6" tree. The selection/transformation process to obtain somatic embryos takes 4-8 months. Thus, the entire process takes approximately 2 years.

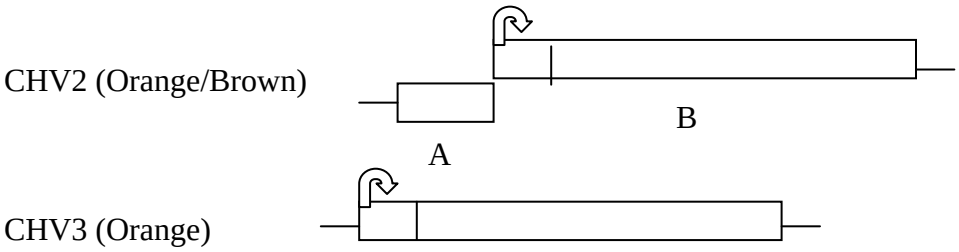
Bradley Hillman, Rutgers University

Viral nomenclature. He reviewed the Family, *Hypoviridae* as follows:

CHV1 (White)



A B



The proper nomenclature of the virus is:

Family: *Hypoviridae*
Genus: *Hypovirus*
Species: *Cryphonectria Hypovirus1*

An acronym, for example, is: CHV1-EP713. The key to nomenclature is based on viral organization, not sequence homology. A correct viral name is based a lot on guesswork if a full-length sequence is not available. Since most viruses are only partially sequenced, naming is done on a 'best guess approach.'

GH2 characterization is finished. In addition to genomic virus, there is a defective RNA, a satellite RNA and a satellite RNA dimer. Size of the particular RNAs are as follows:

RNA1	Full Length	9.8 kb
RNA2	Defective RNA	3.6 kb
RNA3	Satellite RNA dimer	1.9 kb
RNA4	Satellite RNA	0.9 kb

The defective RNA is very different from the genomic RNA; it is only 90% similar. The differences are mostly in the middle bases.

Swiss hypovirulent isolates. He reported on the work of Daniela Linder-Basso. She is characterizing CHV1 isolates from Switzerland. She has found little sequence variation. Most isolates are more similar to Euro 7 than to Ep 713.

Transposon. A transposon is defined as a piece of DNA that can move about the genome. His lab is looking at a transposon that occurs as several-to-many copies in the nuclear genome in *C. parasitica*. This element has been used as a repetitive probe for fungal population studies. While this probe has been used for genotype confirmation, it has only recently been characterized.

pMS5.1- contains a complete copy
-it is moderately repetitive
-used for fingerprint analysis
-apparently few deletion elements present
-AC-like element, not a retrotransposon
-active in the fungus (via northern blots)

The complete transposon is 3.5 kb and contains a large open reading frame that appears to encode for a transposase. Several full copies of the transposon are present in the fungal genome.

Flat mutant. They have found an additional band in the flat mutant, Ep 747ss3. The band is 3176 bp. Linder-Basso is looking at this isolate; there is some evidence for active transposition, based on Southern blots of genomic DNA probed with pMS5.1 or the transposon fragment.

Don Nuss, University of Maryland Biotechnology Institute

Comparative virology. He reported on Baoshan Chen's work with Ep 713 and Euro 7. Comparison of CHV1-EP713 and CHV1-Euro 7 cDNA sequences show extensive sequence identities: 87% to 93% and 90% to 98% at the nucleotide and amino acid levels, respectively. The high level of

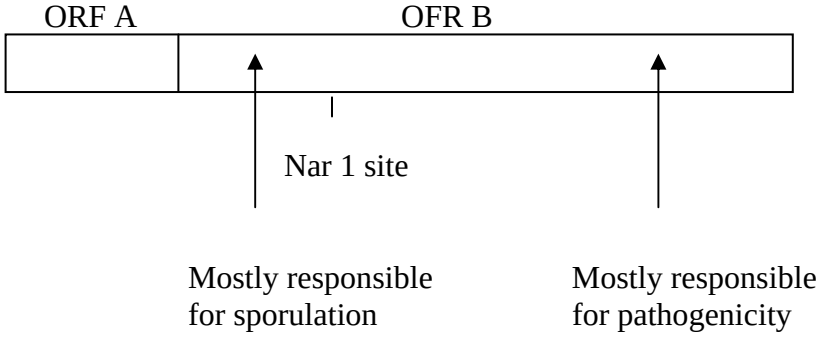
sequence identity between the two hypoviruses allowed construction of viable chimeras. Two chimeric CHV1-EP713—CHV1-Euro7 infectious cDNA constructs, A713BE7 and AE7Ep713, were prepared by precise swapping of the major hypovirus coding domains ORF A and ORF B. He showed slides of a gallery of representative *in vitro* cankers that were formed by virus-free, transfected isolates and selected chimeras. Results indicated that the differences in morphology are due mostly to the viral genome and not the fungal genome. The final magnitude of phenotypic changes, however, were a function of contributions by both genomes. Morphology differences seems to be correlated to ORF B.

The virus component can cause different effects:

- effects the signaling process.
- differential display shows genes that up- or down-regulated after viral infection.

The chimeras have been shown to be very stable. Using domain swapping, it is possible to produce cankers on American chestnut that are quite small, yet produce abundant stromata.

He indicated, via a cartoon, the areas on OFR B that are responsible for sporulation and pathogenicity.



Transgenic field study. Permission was received in 1998 to deploy transgenic strains of *C. parasitica* for population replacement studies in the Meshomesic Forest in CT. Specific risk assessment parameters are:

- Surveying nontarget woody species for infection by transgenics
- Surveying insects for recovery of transgenics
- Determining the effect of transgenic strains on the *C. parasitica* population structure
- Examining the phenotypic variability of recovered transgenic strains.

Two plots were established in the Meshomesic Forest, one control and one transgenic-treated. All vegetation in the control plot was sprayed with a backpack mistblower using either water or transgenic conidia (10^{12} conidia per ml). The area was sprayed 4-6 times per year. While not all the data for 1999 has been analyzed, the following information was presented.

	1998	**1999**
Number of spray dates	4	6
VC Types	25 vc types of 32 isolates	Unknown, 113 collected
Transgenics from insects	0	0/85
Transgenic ascospores	0	48/333

The disease incidence has increased in both plots. No transgenic isolates have been recovered from any other tree species, nor from any insects.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

Meshomesic Forest study. She stated that the chestnut stems are increasing in size in both plots. Perithecia were sampled on 14 September 1999; 15 samples were taken from the treatment plot and 13 from the control plot. She reported that 15 perithecia out of 35 contained ascospores with the Hyg^R marker; these perithecia were from 8 different sprout clumps. She has examined 20 ascospores from each perithecium; to date, 700 ascospores have been examined.

Spore traps have been set out in the plot; petri dishes are changed every 15 minutes. She showed slides of a number of petri plates after incubation. She is having difficulty identifying *C. parasitica* as many fungal colonies, when young, look like *C. parasitica*.

Pam Kazmierczak, University of California at Davis

She reported that Neal Van Alfen has successfully moved his laboratory from College Station, TX to Davis, CA. Although Van Alfen has taken the position of Dean of the College of Agriculture at UC Davis, his laboratory will continue to work on chestnut blight. Their work on hydrophobins continues.

They are specifically working with cryparin, a hydrophobin that may strengthen cell walls in fungi.

Hydrophobins:

- Are produced by many fungi
- Are small molecules (100-125 amino acids)
- Have little sequence conservation
- Have much functional conservation

Known functions of hydophobins:

- Spore dispersal
- Attachment of hyphae to hydrophobic surfaces
- Signal molecules
- Prevention of desiccation
- Emergence of aerial hyphae

Hydrophobins are classified into two types, Type I and Type II. In Type II hydrophobins, there is conservation of spacing of cysteine residues; these molecules are also soluble in ethanol or SDS. She stated that the foam in shake culture is due to cryparin.

A deletion mutant of cryparin was made in *C. parasitica*. Slides illustrated how a drop of water on a culture of Ep 155 did nothing to the aerial hyphae. A similar drop of water on the cryparin mutant, Crp Δ194-7, dissolved the aerial hyphae.

Virulence assays were conducted and no significant differences were noted between Ep 155 and Crp Δ194-7. If virulence is unaffected, what role does cryparin play? She noted a difference in virulent hyphae compared to hypovirulent hyphae. The virulent hyphae was much more "leathery" and difficult to cut. Thus, it was hypothesized that cryparin might have a role in hyphal strength. To test this theory, various isolates of *C. parasitica* were grown, the mycelium harvested, formed into a strip and dried. The strip was then placed in a texture product analyzer to obtain a measurement in force/grams of hyphal strength. Results were the deletion mutant has less texture strength compared to Ep 155.

The deletion mutant was then inoculated into excised stem pieces. She found fewer stromata with the cryparin deletion mutant. After 3 months, Ep 155 produced many stromata, while the deletion mutant had no stromata at all. After six months, all excised stem pieces exhibited stromatal formation. Thus, cryparin may be important for pycnidial production; it may be a pathogenicity factor.

Her conclusions were:

- Cryparin is not necessary for virulence expression.
- Cryparin may be necessary for eruption and stromatal formation.

Mark Double, West Virginia University

Transgenic field studies. Transgenic isolate (Ep 146/pXH9), a genetically engineered brown isolate of *C. parasitica* that contains a full-length cDNA copy of CHV1-EP713 was applied to artificially established virulent cankers in a forest setting. This study was conducted in an effort to determine the most effective method and time of application for spermatization with treatment conidia. Three treatments were compared: transgenic conidia on sterilized bark patches; transgenic conidia in an agar slurry; and, transgenic conidia in a 0.1% peptone solution. Virulent conidia in a 0.1% peptone solution served as a control. When successful spermatization by a transgenic strain occurred, orange, brown and white ascospore resulted from a single perithecium. Two separate experiments were conducted. In both studies, cankers were initiated in May. In the 1997 study, cankers were spermatized in either August, September or October. In the 1998 study, spermatization applications in June, July and August were compared.

In the 1997 study, only August-spermatized cankers yielded ascospores that resulted from treatment conidia. All perithecia collected from September and October-treated cankers, regardless of treatment, yielded only orange ascospores, produced either from selfing or outcrossing to wild-type conidia. Nearly 50% of the perithecia that were treated with the transgenic agar slurry in August were successfully spermatized. Perithecia from the hv peptone and virulent peptone treatments were spermatized by the treatment conidia 2% and 30%, respectively. The bark patch treatment was an ineffective treatment and was omitted from the 1998 study.

In the 1998 study, spermatization occurred with all three treatments on all spermatization dates. When the transgenic agar slurry was applied in June, July and August, perithecia were successfully spermatized by the treatment conidia 74%, 71% and 64%, respectively. The hv liquid results were 77%, 50% and 40%. The virulent liquid yielded spermatization rates of 100%, 80% and 40%, respectively.

Anastomosis versus Transfection. Pathogenicity studies were established on living chestnut stems to compare growth and sporulation of *C. parasitica* isolates when hypoviruses were transmitted via anastomosis or transfection. The study compared six dsRNA-free isolates to those infected with hypoviruses from CHV1-EP713 or CHV1-Euro 7. All thirty isolates were inoculated into healthy American chestnuts in August, 1998. Length and width and a subjective sporulation rating were taken in July 1999. All six isolates in the virulent state produced larger cankers than their hypovirus-infected counterparts. In general, cankers initiated with transfected hypovirus were smaller than those cankers initiated with anastomosed hypovirus. Also, CHV1-Ep 713 hypovirus isolates produced smaller cankers than those initiated by CHV1-Euro 7.

William MacDonald, West Virginia University

West Salem, Wisconsin update. From 1992-94, 186 cankers were treated with a hypovirulent isolate [Wisc. 25-1 (COLI 11-1)] that was created by the introduction of a Michigan hypovirus into the resident West Salem strain. Because hypovirus recovery from new cankers that occurred on trees with previously treated cankers and from new cankers on previously uninfected trees was very low, a second hypovirus was deployed from 1995-97. This hypovirus was transmitted from an Italian strain (CHV1-Euro 7) to the resident West Salem strain, [Wisc. 23-2]. This resident strain was used to treat 479 infections over the 3-year period.

Canker treatment was suspended after the 1997 season. Cankers were sampled in July of 1998 and June of 1999. Approximately 95% of the bark samples collected in 1999 have been cultured and evaluated for their infection status.

New findings for 1999 include:

- 262 trees are now infected with 1250 cankers; 333 new infections and 94 new trees were discovered in 1999.
- 660 cankers were sampled in 1999 of which 233 were new.
- CHV1-Euro 7 continues to be the most commonly identified hypovirus.
- About 10% of the bark plug samples from new infections on newly infected trees contain hypovirus. When calculated on a canker basis, about 30% of these cankers contain a hypovirulent component.

William Jones, West Virginia University

Influence of mycelial age on hypovirus transmission. An orange-pigmented virulent strain (YB-2) of *Cryphonectria parasitica* was used to evaluate the effect of mycelial age on hypovirus transmission *in vitro* and *in vivo*. On June 25, 1998, YB2 was used to initiate cankers that were vegetatively compatible with a brown-pigmented strain (80-2C). Each month, the expansion of cankers was traced with a permanent marker. On October 21, 1998, cankers were challenged at the leading edge with mycelial plugs of 80-2C [CHV1 (80-2)]. Control cankers were treated similarly except 80-2C was hypovirus free. Starting on November 27, 1998, the traced canker rings were sampled monthly for 7 months using a 2-mm bone marrow biopsy tool to remove bark plugs. Bark plugs were cultured for *C. parasitica* and colonies scored for their origin and virulence based on morphology and pigmentation. The total percentage of hypovirulent (HV) isolates from each of the monthly samples taken from November to May was 3%, 24%, 29%, 12%, 14%, 23%, and 42%.

A second component of this study was to evaluate hypovirus transmission *in vitro*. On June 8, 1999, YB2 was used to initiate 420 *C. parasitica* colonies on potato dextrose agar. Groups of 60 colonies were challenged with 80-2C [CHV1 (80-2)] after 0, 1, 2, 3, 4, 5, and 6 weeks of growth. Isolates were co-inoculated, challenged at the leading edge after 1 or 2 weeks of growth, or challenged opposite the initiation point if the mycelium had filled the plate. Starting one week after each of the challenges, 12 mycelial transfers were made from 10 plates each week for 6 weeks. The cultures that resulted were scored for virulence and their origin based on morphology and pigmentation. The average percentage of isolates that were recovered from cultures each week after challenge that exhibited hypovirulent morphologies were 78%, 18%, 6%, 0%, 0%, 0%, and 0%. A related study to this compared the recovery of HV isolates when challenged by 80-2C [CHV1 80-2] to those challenged with 80-2C [CHV3 (COLI 11-1)]. A control was challenges using 80-2C free of hypovirus. Treatments consisted of co-inoculations (90 colonies), challenges at the leading edge of one-week old colonies (90 colonies), and challenges behind the leading edge of the mycelium (90 colonies). No significant differences were found between the two hypoviruses but significant differences were found between challenges made at the leading edge (34% of the orange pigmented samples were HV) and challenges made behind the leading edge (5% of the orange pigmented samples were HV).

Dipnath Baidyaroy, Michigan State University

Mitochondrial hypovirulence. Upon further investigation of hypovirus-free strains from the Kellogg Forest that demonstrated the transmissible hypovirulence phenotype, he was able to identify the causative mutation in the mitochondrial DNA of these strains. The hv strains contained an insert of unknown function and origin, named 'InC9' in the small subunit rRNA gene that was not processed properly from the premature rRNA. This resulted in the strains being deficient in mitochondrial ribosomes and hence, in mitochondrial protein synthesis. However, apart from the InC9 element, these hv strains often contained a circular, plasmid-like element that was partly homologous to the mitochondrial DNA itself. This circular DNA, named pleC9, (plasmid-like element) also appeared to be an infectious element and could be detected in the majority of strains isolated from the Kellogg Forest

stand. The element pleC9 had an adverse effect on growth phenotype but did not confer hypovirulence by itself. Sequence data showed pleC9 is 1364bp; it is G/C rich.

An isolate from Kellogg Forest, KFC9-E6, is unstable. The isolate yields colonies with varying morphologies (slow-growing, fast-growing and those that do not maintain a pattern, alternating between fast and slow). While the morphologies were different, a DNA probe found all isolate types to be similar at the molecular level. He grew the inoculated the different morphology types into apples and excised chestnut stems. The data are:

Isolate	Pathogenicity in Excised Stems (cm)
EP 155	6.71
KFC 9-Fast	3.43
KFC 9-Fast/Slow	0.10
KFC 9-Fast/Slow/Fast/Slow	0.60

The slowest growing isolate had a very amplified amount of the pleC9 element. His speculation is there is a nuclear signal for the plasmid-like element that is triggered in the nucleus, although he has not seen any differences in the nucleus.

His conclusions were:

- pleC9 element cannot independently trigger hypovirulence.
- pleC9 DNA can adversely affect growth rates of the fungus.
- Accumulation of the pleC9 DNA results in concomitant loss of mitochondrial DNA.
- The element is infectious and is probably transmitted cytoplasmically.
- Inc9 element is probably the primary cause of hypovirulence in the strains located in the Kellogg Forest stand.

Andy Jarosz, Michigan State University

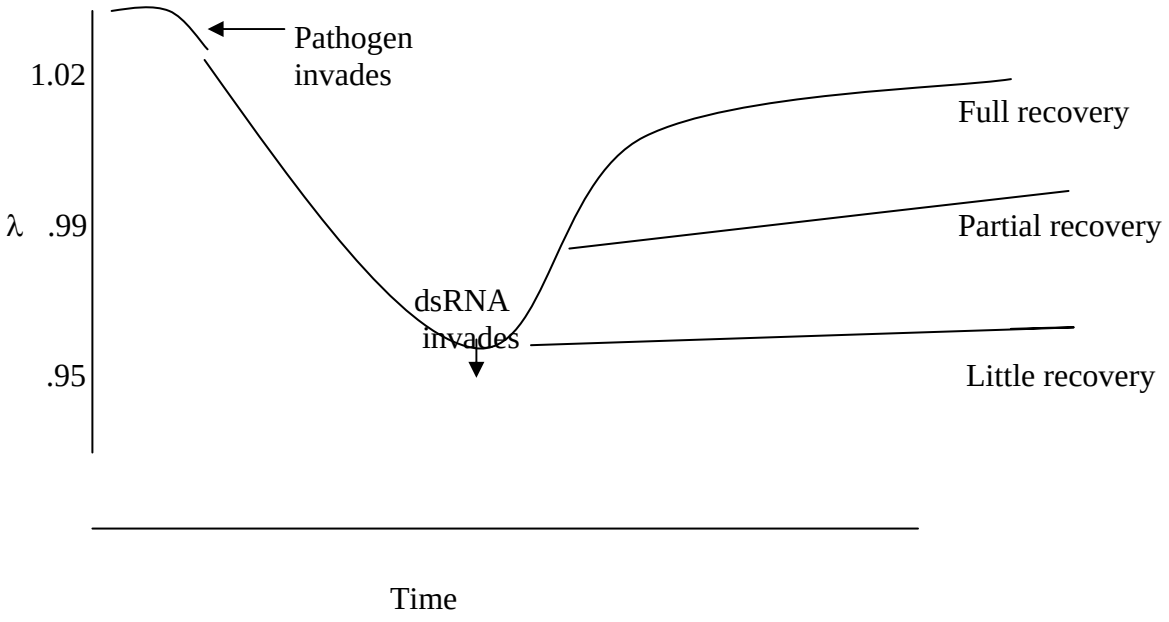
His main objective was to determine if there was an interaction between the presence of dsRNA and branch size on the survival of infected branches. He speculated that when the virulence of dsRNA-containing isolates is high, dsRNA spreads but trees still die. Conversely, when virulence of dsRNA-containing isolates is low, then hv isolates do not spread. He believes there should be some optimum level of virulence for dsRNA to work effectively.

There are some recovering populations of American chestnut in Michigan. Even in these sites, there are small branches that are dying back. Thus, hypovirulence is actually size-dependent. His hypothesis is: the effect of dsRNA on branch survival depends on the size of the branch or, positive effects of dsRNA are more evident on larger branches. In order to test this theory, he took a COLI isolate, with and without dsRNA. He chose 20 branches, 10 of which were inoculated with the dsRNA-containing isolate; 10 were inoculated with the dsRNA-free isolate. Healthy, small (< 2 cm diameter), medium (2-4 cm) and large (> 4 cm) branches were inoculated in July, 1997. Nearly all small branches have died after 2 years, regardless of dsRNA treatment. In contrast, nearly all large branches have survived, regardless of dsRNA treatment. Only for medium size branches has the presence of dsRNA increased branch survival.

Anita Davelos, Michigan State University

She reported on chestnut demographics in Michigan. The main objectives of her work were: (1) determine how disease alters host demographics; and, (2) evaluate the extent of dsRNA-mediated recovery in American chestnut populations. Six chestnut populations (two healthy, two with blight and two with blight and dsRNA) were monitored from 1996-1998. She posed a hypothetical graph of population recovery.

Time Growth Course of Chestnut Population Growth Rates in Naturalized Populations



Matrix projection models of population growth rate and size class distributions revealed that chestnut blight infections increased the probability that large trees may be reduced in size and increased the probability of smaller trees reproducing, regardless of the presence of dsRNA. Population growth rates (λ) varied among sites and among years. However, the relative rankings, with non-recovering populations having the lowest growth rates over two census periods, indicated that disease is indeed having a negative impact on chestnut population growth rates. The intermediate rankings of recovering populations indicate that the presence of dsRNA can promote ecological recovery of American chestnut populations. The pattern for diseased populations being dominated by intermediate-sized individuals at stable stage, while healthy populations are dominated by small individuals, was further indication that disease is influencing American chestnut populations.

Her data indicate that in non-recovering populations, populations in the 1-10 cm class size are most affected. This suggests that biological control efforts should concentrate on trees 1-10 cm dbh.

A summary of population growth rates is as follows:

- There is variation among years of tree survival.
- The lowest growth rates were for non-recovering populations.
- The growth rates for health and recovering populations are similar.
- dsRNA can aid in recovery.

Colin McKeen, Canadian Chestnut Council

The Canadian Chestnut Council was given an award earlier in October by the Carolinian Canada Group for their work with chestnut over the past 15 years.

He indicated that American chestnut can be found in Canada in counties that border Lake Erie as well as a few inland-counties. In Canada today, McKeen is dealing with remnant populations of American chestnut. Most trees can be found along fence rows or in larger wooded areas. He conducted a survey 15 years ago and found eight sites that contained American chestnut. In cooperation with Dennis Fulbright of MSU, they identified dsRNA in seven of the eight sites. The Arner site did not contain dsRNA. He showed slides of the Arner tree between 1984 and 1999. The tree is now 33" dbh and has many cankers. While the cankers do not contain dsRNA, they are "healing-like." He isolated

from the Arner tree and tested the effectiveness of that isolate by inoculating cankers on other trees. After one year, those inoculated cankers showed significant callus production. However, after 12 years, the challenged tree is mostly dead.

At another chestnut site, east of Windsor, Ontario, he found cankers that contain dsRNA similar to GH2. The best example of closing cankers can be found near the Vienna Cemetery where trees are 40'-50' tall.

He also showed slides of three tall trees in Ontario that have died suddenly. Mortality was not due to chestnut blight; he suspects *Phytophthora* sp.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

Graft union fungi. She reported on the other problems Greg Miller has in his Ohio orchard. In addition to graft incompatibility (discussed earlier), he has fungal infections in the graft unions. Two years ago, she suggested Miller surface sterilize the graft unions. Upon grafting, he took a small sliver of bark that was used to inoculate Granny Smith apples. Anagnostakis identified many fungi that grew in the apple tissue. The most common isolates were inoculated into excised chestnut stems. The fungi that were identified were: *Botrytis, Fusicoccum, Cytospora, Myriellina, Chaetomella* and *Melanconium*. She believes that *Melanconium castaneum* is the fungus responsible for graft union contamination; it is not a strong pathogen. They are screening pesticides to combat this problem.

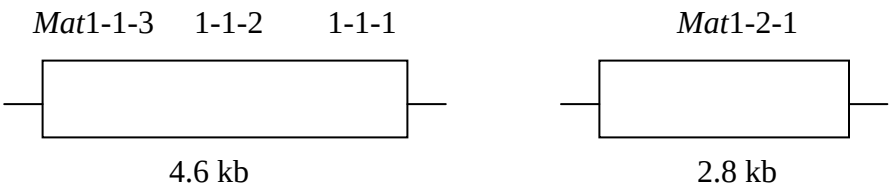
Lockwood Farm. A orchard of American chestnut trees was planted by Richard Jaynes at CAES in 1976. By 1978, the trees were cankered and subsequently treated with a mixture of hv strains from Italy, France and North America. Treatment continued for four years. The trees are now 25 years old. Some of the trees remain healthy while others have died back. She recently isolated hypovirulent isolates from some cankers. She also isolated a "flat strain." When a virulent isolate is challenged with the flat strain, it converts to a white phenotype. In cankers from four large trees, she has isolated new vegetative compatibility types from the larger cankers. Sexual recombination continues in the orchard.

Dennis Fulbright, Michigan State University

He reported on the County Line site, located in northern Michigan. In 1980, the stand was classified as a recovering site. By 1985-86, it was a premier recovering site. In 1999, the chestnut trees have grown so well that the canopy has closed. The landowners took seed from the County Line site and planted the nuts at their home, two miles away. This site, known as the Rau site, currently has 30 trees, half of which are infected with *C. parasitica*. Some of the killed trees have resprouted. Studies were initiated in 1996 as no recovery was evident. All *C. parasitica* strains isolated from the site were in the same vc group as that of the predominate vc group at the County Line site. All but one of the strains was considered virulent. The single hypovirulent strain was recovered from a lethal canker and the isolate was not infected with dsRNA. Healing cankers have increased at this site although dsRNA has yet to be detected. The strains have not been assayed for mito-chondrial hypovirulence. It is surprising that there is no COLI virus at this site since it is only two miles distant from the premier recovering site. Recovery is occurring in the absence of dsRNA.

Michael Milgroom, Cornell University

Mating type idiomorphs. Previously, he had identified sequences specific to each mating type (*MAT*) idiomorphs in *C. parasitica* and developed a PCR method for determining mating type. Recently, he has completed the cloning and sequencing of both idiomorphs. The size and organization of *C. parasitica* idiomorphs are very similar to other pyrenomycetes (*Neurospora crassa* and *Podospora anserina*). The mating type nomenclature needs to be changed to *MAT*1-2 and *MAT*1-1 to be consistent with idiomorphs in other fungi.



Vegetative compatibility types and virus transmission. He is continuing to compare the vc types in the United States and Europe. Vegetative compatibility testers were developed for European populations of *C. parasitica* in cooperation with Paolo Cortesi (Milan).

Comparison of vc types in eastern U.S. to European Union (EU) vc types

U.S. Population	Total # vc types	# Common with EU	Non-EU
Finzel, MD	26	23	3
Bartow, WV	30	22	8
Depot Hill, NY	34	20	14

There are 64 vc types, as determined by 2^6 for two alleles at six loci. There is more *vic* gene diversity in the U.S. than in European populations.

His data show that virus transmission is inhibited with increasing vc types. There is a correlation between biocontrol and vc types. In preliminary analyses at the population level, the average transmission probability within Italian and U.S. populations is highly correlated to vc type diversity. In contrast, incidence of CHV1 in Italian populations is not correlated to predicted average transmission.

The following is a table of very rough estimates; this data has been updated but not presented.

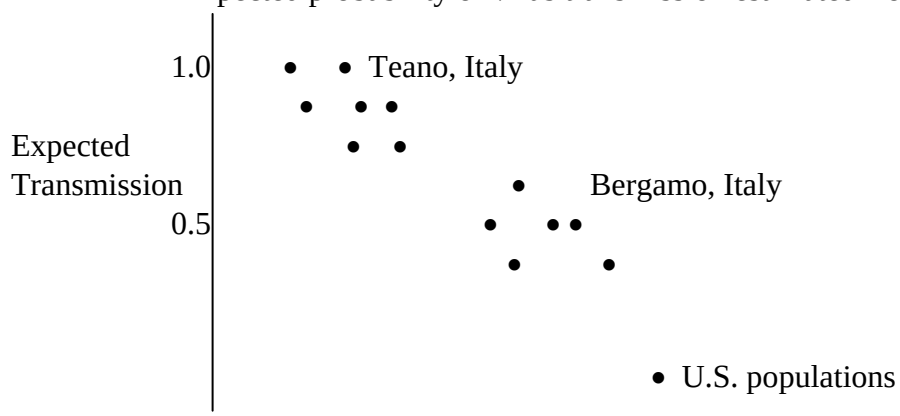
Effects of each vic gene on virus transmission

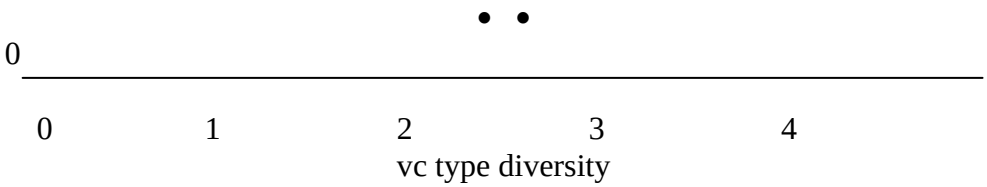
***vic* locus**	**Probability of Transmission**
vic 1	0.02/0.98*
vic 2	0.07/0.20
vic 3	0.68
vic 4	1.0
vic 5	1.0
vic 6	0.11/0.38
vic 7	1.0/0.32

*0.02=when donor has allele 1-1 and recipient 1-2
0.98=when donor has allele 1-2 and recipient 1-1

The goal is to develop a model to predict virus transmission given any one of the 64 vc types. He showed the expected probability of viral transmission in three populations.

Expected probability of virus transmission estimated from vc type distribution





The question is, 'Why don't viruses spread in U.S. populations?' Vegetative compatibility diversity is not the only factor. One possible explanation is there might be some threshold level of vc diversity that, when reached, diversity does not increase. All claims of hypovirulence in Europe are not based on very hard data. Bergamo, Italy has the highest diversity of any of his Italian plots. Perithecia can be found in Italian populations and the 'selfing rate' is similar to that in the U.S. (~25%). Viruses do not seem to effect vc diversity.

CHV1 in Asia. There are two major groups of CHV1 in Europe, those found primarily in France and those found in Italy, Switzerland and the rest of southeastern Europe. In order to determine the origin of European strains, nucleotide sequences of CHV1 isolates from Asia, Japan and Europe were analyzed.

Incidence of hypoviruses in China

Population	Number	CHV1	CHV2
China			
Random	257	1	2
Non-random	23	22	1
Japan	605	20	0

Using a 1096 bp sequence from ORF B, approximately 10 CHV1 isolates from China, Japan and Italy were analyzed A phylogenetic tree was obtained from the isolates with the results:

- Everything from Italy was on one clade.
- Japanese isolates were clustered alone.
- China, Spain, Germany and France were clustered together.

Sequence similarity data indicate that CHV1 isolates from China are virtually identical to each other and to Ep 713. After discussions with Anagnostakis as to the shipment of hypovirulent isolates to Asia, it was concluded that the CHV1 isolates from China are the result of viral introduction from France, via Connecticut.

Robert Doudrick, USDA-Forest Service, Asheville, NC

Genetic variation in American chestnut. He reported on Tom Kubisiak's work; he is assessing the genetic diversity of wild populations of American chestnut collected from across the natural range. Currently, DNA has been extracted from twigs collected from 21 sites, as listed in the table below.

Locations	**# of Populations**
Southern Ontario	1
Maine	1
Massachusetts	1
New York	2
Connecticut	2
Pennsylvania	3
Maryland	1
West Virginia	2
Virginia	3
Kentucky	1

North Carolina	2
South Carolina	1
Georgia	1

DNA from a total of six populations (one from Virginia, one from West Virginia, one from North Carolina, one from Massachusetts and two from Connecticut) have been sent to Dr. Robert Bernatzky at the University of Massachusetts. Bernatzky is currently genotyping all six populations for 10 RFLP loci.

Mapping resistance loci. The American Chestnut Foundation is focusing on three sources of resistance to *C. parasitica* in their backcross breeding program: Graves ('Mahogany'); Nanking; and, Clapper. Their initial mapping work has indicated that the Graves source of resistance has at least some genes different from those of the Nanking source. Their main goal is to extend the genomic mapping work and map regions conferring resistance in the third main source of resistance, Clapper.

Genetic markers and vic loci. They hope to identify genetic markers tightly linked to vic loci in *C. parasitica*. Once identified, these markers can be used to study vc group and vic allele frequencies in natural populations. To date, they have place five vic genes on a genetic linkage map of *C. parasitica*. The marker mapping work is being done by Michael Milgroom who is currently developing additional markers (AFLP) in an attempt to saturate the genome with markers.

Paul Sisco reporting for Robert Bernatzky, University of Massachusetts

Mapping efforts. The RAPD work went well with the backcross samples but not with the F_1s. The RFLPs are being done by a parental screen. He is doing a panel of 15 genotypes, consisting of Chinese, American, Graves and Clapper. He has a freezer of 200 cDNA clones. Two restriction enzymes were being used, EcoRI and HindIII. Using one marker, he found the same morphology in all three Chinese, and the same for the six American samples. The Graves/Clapper morphologies were similar to that of the Chinese.

Genetic diversity study. Tom Kubisiak sent Bernatzky 6 populations of American chestnut twigs to examine genetic diversity. Bernatzky has pooled samples from 60 twigs collected at each site. To date, he has examined two samples each from Massachusetts and Virginia. Probes that are close to the resistance loci are being used. For some probes, the patterns are monomorphic. For other probes, there is a great deal of diversity. His data so far indicate:

- 90% of DNA diversity is within a site.
- 10% of the DNA diversity is between populations.

The key difference may be things like tree diameter. There are some markers that are unique to each site. They want to explore the 10% that show differences between populations to see if markers can be identified that pick up site differences.

What does this mean to adaptability? There are important adaptive differences that these tests do not pick up.

Business Meeting

Sandra Anagnostakis was elected secretary for 2000-2001. Chairman for 1999-2000 is Hill Craddock. A possible site for next year's meeting is the freshwater aquarium in downtown Chattanooga. The TVA is another possible site. The meeting date that was the consensus of the group was October 27-29, 2000. After the 2000 meeting site was discussed, the USDA plaque was passed from Fulbright to Craddock.

John Anderson was asked to present an update on the West Nile virus. To date, seven individuals have died from this encephalitis-related virus. His 20-minute report covered the history of the virus and their attempts to identify the outbreak in the NY/CT area.

Dennis Fulbright acknowledged Andy Jarosz for his efforts in arranging the meeting. Paul Sisco led the NE-140 members in thanking the Michigan State University group for their efforts. The meeting adjourned at 11:45 am. A roundtable discussion on the West Salem, Wisconsin stand followed the general meeting.