NE-140 Technical Committee Meeting
Biological Improvement of Chestnut (*Castanea* spp.)
Best Western Inn at Hunt's Landing, Matamoras, PA
September 18–20, 1997

Attendance:

Connecticut:	Sandra Anagnostakis, Phillip Gordon, John Anderson
Georgia:	Rodney Robichaud
Kentucky:	Lou Shain
Maryland:	Don Nuss, Baoshan Chen, Shin Kasahara, Nobuhiro Suzuki
Massachusetts:	Robert Bernatzky
Michigan:	Andrew Jarosz, Helmut Bertrand, James Bier, Anita Davelos, Dipnath Baidyaroy
Mississippi:	Robert Doudrick, Thomas Kubisiak
New Jersey:	Bradley Hillman
New York:	Michael Milgroom, Yir-Chung Liu
Tennessee:	Scott Schlarbaum, Hill Craddock
Texas:	Neal van Alfen
USDA-CSREES:	Robin Huddle
Virginia:	Fred Hebard
West Virginia:	William MacDonald, Mark Double, Clarissa Balbalian, Paul Chaloux, William Jones

The meeting was called to order at 8:10 pm September 18, 1997 by Chairman van Alfen. John Anderson, Administrative Advisor, said that one of the most important tasks of the committee is the renewal of the project. He then proceeded to read a letter, dated 30 July 1997, from the Northeast Experiment Station Directors and an attached resolution of recognition from Dr. Rosemary Haggett, Dean and Director of the West Virginia Agriculture and Forestry Experiment Station.

Dr. Anderson went on to report that regional research does not stand on past laurels, but on proposed interdependent research. The new project has yet to be approved by the Northeast Directors and there is some opposition to NE-140. It has been a long-running project and some directors think projects should come to an end so money can be made available for other, newer projects. The regional research directors initially voted down NE-140 at their February meeting. The Northeast Station Directors argued against the rejection.

Sandra Anagnostakis put a new proposal together and Anderson sent out the new version to several noted plant pathologists. He received three responses; their comments and criticisms will be included in the final proposal. The committee must address the reviewers and how their concerns will be addressed.

The time line for the new proposal is as follows:

- An initial draft of the proposal has been prepared and distributed to all members and reviewers.
- Letters have been sent to all Experiment Station Directors with regard to the amount of time they will allot their personnel to work on NE-140 related projects.
- A rewrite then needs to be done and this will be mailed to the Director of each institution to get them to sign off.
- The proposal will need to be sent to the Northeast Directors three weeks prior to their meeting in March, 1998.

Anderson went on to say that some directors feel that the USDA-FS should be doing work on chestnut, not members of Northeast Experiment Stations. Anderson's argument was that

during the past five years, members of NE-140 have published 158 research papers and information publications while the USDA-FS did not publish a single paper on chestnut. All work in the United States on chestnut and chestnut blight is being done by members of NE-140.

It was Anderson's opinion that NE-140 still has a battle on its hands. A proposal needs to be submitted that the Northeast Directors cannot refuse. He is optimistic but cautious that the moment of success has passed and we must look to the future. The catch word, "interdependency," is the key to the success of renewal.

Meeting minutes are limited to three pages as a project report. The NE-140 committee report must be out by March, 1998. The report should be limited to 3 pages, not including the publication list.

Chairman van Alfen urged all members to come to a consensus about the new proposal.

Robin Huddle, USDA–CSREES

She passed along greetings from Dr. Jack Barnes, past USDA-CSREES representative. Barnes is now retired, although he is still working as a continuing annuitant.

She then presented the latest information from Washington, DC.

- Hatch funds should be stable for the upcoming fiscal year, although the final budget is not yet finished.
- There is new, separate mandatory funding of $780 million for future agriculture and food issues.
- The fund for rural America will be approximately $100 million.
- The USDA-ARS will be reviewed.

Many regional research projects have been terminated lately. She encouraged new and creative ideas for the next 5 years. The new NE-140 project must show how members have cooperated in the past and make sure the proposal is written so it is understood easily.

Sandra Anagnostakis then reported on the awards ceremony at the USDA in Washington, DC where NE-140 was awarded the Secretary of Agriculture Honor Award. She passed out extra programs from the ceremony along with some photographs and coasters.

William MacDonald passed out flyers announcing the International Chestnut Congress in Bordeaux, France in October 1998.

The data presentations are organized by Regional Project objectives and presented by station.

<u>OBJECTIVE 1.</u> To investigate the genetic determinants of hypovirulence (H) and their effects on the establishment and dissemination of the pathogen in the forest ecosystem.

Baoshan Chen, University of Maryland

He talked about comparative virology, with respect of introducing the viruses from Euro 7 and EP 713 into EP 155. These viruses are both classified as CHV-1 viruses. (Euro 7 is from Florence, Italy and EP 713 is from the Pyrenees in France).

When Euro 7 and EP 713 are plated onto PDA, Euro 7 produces abundant spores while EP 713 lacks spores. The virus determines the phenotype of the fungus. He then put viruses into EP 155 and Euro 7, virulent. Euro 7 in EP 155 will not produce spores on PDA. This is the first indication that the right fungal host is needed to get production of spores with the same virus.

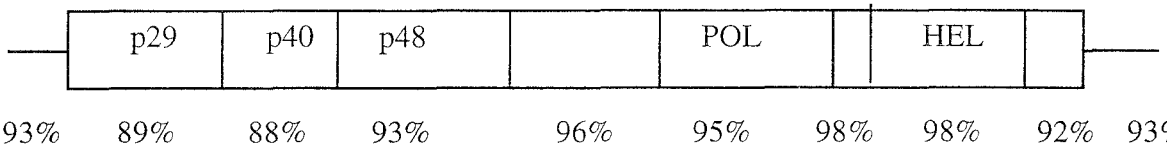
He converted Euro 7, virulent with Euro 7 and EP 713, and the 713 virus seems to be dominant, phenologically. Even though a fungus is infected with one virus, it can be infected with a second virus.

Excised chestnut stems were inoculated with the following:

EP 155, EP 155 [EP 713], EP 155 [Euro 7], Euro 7 (v) and Euro 7 (v) [Euro 7].

EP 155 and Euro 7 (v) both grew and sporulated well. EP 155 with the Euro 7 virus produced little or no growth and no sporulation. Euro 7 (v) with the Euro 7 virus also grew well and sporulated.

The structure and sequence of EP 713 is well documented. Bao has been working on cloning and sequencing the Euro 7 virus; the construction of a full-length Euro 7-derived hypovirus is near completion. Euro 7 is very similar to EP 713; the amino acid sequences for the Euro 7 hypovirus, compared with CHV1-713, is 99% with ORF B and 88% with ORF A. The following figure shows the percent identity with CHV1-713.



Why are the two viruses so close, molecularly, but so different, biologically? He displayed the following table:

Hypovirus–Fungus Interaction

Hypovirus Virulence		**Fungal Susceptibility** EP 155 (rr)	Euro 7 (RR)
Benign	ab	–	–
Semi-hypovirulent (i.e. Euro 7)	aB	++	+
Hypovirulent (i.e. EP 713)	AB	+++	+++

Benign viruses do not cause disease in the fungus; there is dsRNA but no hypovirulence. Mutations may occur over time until, at some point, the virus causes a disease of the fungus. He hopes to determine the virulence region with domain swapping.

Comments: Brad Hillman said that there are benign viruses in the southern U.S.; he cited SR2 in West Virginia. Anagnostakis said there are no benign viruses in Connecticut. Gordon commented that Connecticut was glaciated so it would be expected that there would be more diversity in the southern U.S. compared to New England.

Chen reported that Shaojian Gao has been working with EP 721; it hybridizes to EP 713. He is continuing his work on the EP 721 genome.

Mark Double, West Virginia University

He reported on virulence study initiated in July 1997. Several wild-type isolates were infected with the Euro 7 hypovirus. The following is a summary of (L+W)/2 data collected after 11-weeks:

Isolate	**(L+W)/2 (cm)**
EP 155	8.81
Euro 7, virus free	9.06
Schomberg, virus free (WI)	8.72
Bockenauer, virus free (WI)	8.4
Windam Rock, virus free (VA)	7.93
Jeremy Run, virus free (VA)	7.50
Euro 7	7.12
EP 155 {Euro 7] Maryland's isolate	5.84
EP 155 [Euro 7] WV's isolate	7.03
Schomberg [Euro 7]	8.31

Bockenauer [Euro 7]	7.66
Windham Rock [Euro 7]	3.75
Jeremy Run [Euro 7]	4.34
EP 713	5.65
Schomberg [COLI]	2.37
Bockenauer [COLI]	2.99

Comments: Anastomis is not a clean system to transfer viruses as there is a significant contribution of the fungal host. Transfection sets with and without virus are needed.

Helmut Bertrand, Michigan State University

He reported that the Arn tree in southern Ontario is improving. Close by, however, are other infected trees that seem to have lethal cankers. This tree is a good example of mitochondrial hypovirulence.

Alternative oxidase is the diagnosis for mitochondrial hypovirulence. It causes significant effects on fungal growth. He conducted apple and excised stem inoculations with a variety of isolates and found that laboratory induced strains were more debilitated than isolates from nature.

Knowing that there are mitochondrial defects, more information is needed about mitochondrial DNA. It is very large, about 160 kb. The first important aspect is it accumulates a plasmid-like element. Two-dimensional gels were run to look at the structure of the DNA. There is a high accumulation of a small rRNA. A plasmid was found, pCRY1; it is found in a number of strains. Some *C. parasitica* strains that are unstable but have no virus may contain this plasmid. The plasmid, 4.2 kb, encodes a DNA polymerase and is related to the benign Fiji and LaBelle plasmids of Neurospora. Although pCRY1 slightly depresses the virulence of *C. parasitica*, like Fiji and LaBelle, it may be benign because there is no indication that it induces deleterious mtDNA mutations. This plasmid is very invasive and it is transmitted via anastomosis.

Dipnath Baidyaroy, Michigan State University

A strain of *C. parasitica* was isolated from the Kellogg Forest, KFC9. This strain exhibits slow growth, high levels of alternative oxidase activity and a hypovirulent phenotype that is cytoplasmically transmitted; it lacks dsRNA. He isolated mtDNA from EP 289 and found quite a few RFLPs between EP 298 and KFC9. He found a 10.2 kb section associated with both strains and thought this might be responsible for the phenotype. It is not intronic and sequencing did not tell why there is hypovirulence. Since spontaneous and induced hypovirulence phenotypes often are associated with rearrangements affecting the mitochondrial subunit ribosomal RNA, they sequenced the srRNA gene and found that it is exceptionally long and contains four introns, each spanning about 2-kbp.

Brad Hillman, Rutgers University

He reviewed the members of the Hypoviridae family

CHV1 European origin, confers white phenotype, 2 ORF, 12-13 kb
CHV2 New Jersey origin, confers thin brown phenotype, 2 ORF, 12-13 kb
CHV3 Michigan origin, confers abnormal margin, 1 ORF (ORF B), no ORF A homoglog, 10 kb
WV-type, confers no apparent phenology change (i.e. SR2)

NB58

- 2 complete cDNA clones have been made; one PCR and one traditional.
- Total of 48 confirmed differences from published sequence in the 4 kb region.
- 41 confirmed difference from published sequence in the traditional clone.
- 17 confirmed difference from the PCR clone.

Italian dsRNAs
- 12 isolates from Teano, Italy have been characterized.
- 190nt sequenced from each: ~800nt near the 5' end, ~1100nt near the 3' end.
- There are few differences among the isolates.
- CHV1-EP 747 appears closer to CHV1-EP 713 than to the Teano isolates (EP 747 is from northern Italy.

GH2 dsRNA hybridization

band 1 9.8 kb
band 2 3.7 kb
band 3 2.0 kb
band 4 1.0 kb

Hillman has completed the characterization of the 5' end. Chris Smart had said there was no ORF B homoglog, so Hillman finished sequencing the 5' terminus and it looked a lot like EP 713.

SR2 dsRNA (Savage River, Maryland)
- It causes little hypovirulence or alteration in colony morphology.
- Single dsRNA ~9-10 kb.
- Hybridizes to many dsRNAs from northeast U.S. and Michigan.
- Sequence similarity is closest to GH2, phylogenetically.
- As a member of the Hypoviridae, it will most likely end up with CHV3, unless it ends up in its own category.

NB58F (New Brunswick, NJ, virus-free)
- It resists infection by members of the Hypoviridae.
- NB58, NB58F and NB58-19 all have the same fingerprint but 58F contains the "B" chromosome.

He is looking at genes that may be involved in this phenotype. He looked at gene, A3-13, and did sequence analysis. A phylogenic tree was constructed and its closest relative is β-glucanase from sea urchin (*S. purpuratus*). It is even closer to sea urchin than to other fungal β-glucanase.

Neal van Alfen, Texas A&M University

He is interested in several projects:

1. How does a virus affect a fungus? To understand this question, products must be identified and then examined for what they do.
2. Replication of viruses. Replication takes place on small membrane vesicles.

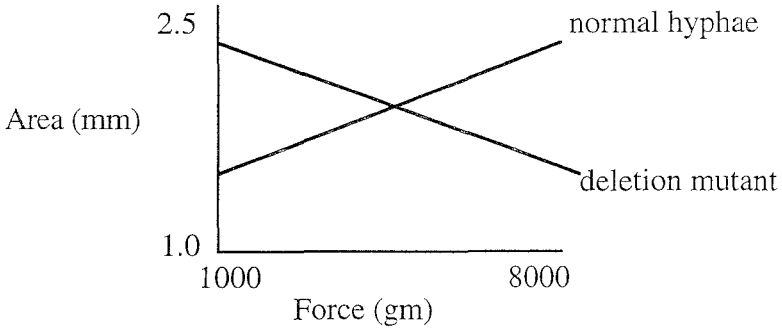
A virus affects a fungus by proteins that are produced. The following proteins are all down-regulated:

Protein	Signal Peptide
Cryparin	MQF51
Laccase	MP5FF
Pheromone	MRFTA

What is a commonality among all 3 products? They are all processed in transport by signal Kex2 processing. All are directed to a late golgi vesicle for processing before selection. Cry is present in the same vesicle fraction that supports virus replication. Therefore, does virus replication interfere in any way with the above products? If virus is affecting transport, is there an accumulation of these proteins in the cytoplasm? One way to look at this problem is to use pulse

labeling and look at the rate of secretion. At this point, preliminary results support the hypothesis that there is an accumulation within the cell.

Are virulent hyphae any different from hypovirulent hyphae? How do you quantitate "toughness" of hyphae? He collected masses of hyphae, mashed them down and put them in a machine that applies force until hyphae break. He examined hyphae from a normal strain and hyphae from a strain with a deletion mutant.



There is no relationship between area and force with the deletion mutant. With Cryparin, there is evidence that it is important in cell wall strength.

Pheromones:

Mat-1 prepheromone

Pheromones are secreted to the outside of the cell to a receptor through a map kinase pathway. The pheromone will inhibit conidial germination down to 10^{-9} M. This has implications if you have a small peptide that decreases germination at low levels. This may aid in biocontrol.

OBJECTIVE 2: To study the ecology and physiology of *Castanea dentata* and ecology and dissemination of the pathogen, *Cryphonectria parasitica*.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

Transgenic Hypovirulent Plots. She conducted experiments with recombinant strains produced by Don Nuss in two separate experiments. The first experimental plots were in Sharon, CT. The deployment of the recombinant strains in this first field test was: (1) mycelial plug deployment and, (2) conidium-painted deployment. She is continuing to monitor these plots but samples from new cankers this spring did not include any that were transgenic.

Her second transgenic field test is in the Meshomasic State Forest near Portland, CT. An area 200 x 400' with good chestnut was cleared of all competing vegetation. A fire occurred on this site in 1986, giving rise to two generations of dying and sprouting chestnuts. Two plots (50 x 75') have been chosen; there are 23-24 chestnut stems/plot. She is maintaining clearing as per Gary Griffin. Isolates were collected and typed for vegetative compatibility. Isolates of the three most common v-c types were transformed by Don Nuss using DNA mediated transformation with the plasmid used in the initial field experiment, and with a plasmid containing a modified CHV1-EP 713 cDNA. The modification involves deletion of the coding domain for viral protein, p29, which has been shown to contribute to virus-mediated reduction of fungal sporulation. This should allow the transgenic strains to produce more conidia. One field plot will be sprayed with water while the second plot will be sprayed with transgenic strains of 10^7 conidia/ml using a four gallon back-pack sprayer. Plots will be sprayed twice a year (spring, summer), or more often if warranted. Plots will be monitored by examining isolates from natural cankers, spore traps and insects.

Peroxidase Studies. Peroxidases are enzymes involved in lignification and possibly linked to tree resistance. She has been sampling twigs monthly, freeze drying them and then assaying for proteins. She has found that total peroxidase activity varies with season. She examined the following isotypes:

A1	Michigan or Wisconsin trees	only A isotype of peroxidase 2
A2	Scientist Cliffs	
C1	Chinese-Mahogany	has lowest activity
J1	Japanese-timber type tree	only B allele of peroxidase 2
J2	Japanese-orchard type tree	only B allele of peroxidase 2

Can any of the peroxidase types be correlated to resistance? Some isotypes come and go. The B band occurs in one Chinese and in one American. She found enormous amount of one band in Chinese chestnut for both control and canker tissue while American chestnut had enormous amounts of one type in control tissue but none in canker tissue. Peroxidase bands cannot be used to differentiate American from Chinese chestnut directly; only inferences can be made with certain band combinations.

Donald Nuss, University of Maryland

He questioned three aspects of hypovirulence: (1) transmission through ascospores; (2) persistence; and (3) dissemination. He reported on his cooperative study with Anagnostakis' first field experiment.

Virulent test strains

EP 154	mat1-1	v-c39	orange	virulent
EP 392	mat1-2	v-c39	orange	virulent

Transgenic strains

EP 146/pXH9	mat1-2	v-c9	white (brown)	hypovirulent
EP 155/pXH9	mat1-1	v-c40	white (orange)	hypovirulent

Combinations were: EP 154 x EP 146/pXH9 and EP 392 x EP 155/pXH9. Both sets were mating type compatible and v-c compatible. There were two plots, one painted and one plug inoculation. Pairs of trees with initiated cankers were in the study, but only one tree/pair was treated with hypovirulent isolates.

Perithecia were recovered on September 16 and November 8, 1996. The end result of this experiment was that most trees died.

Plot 1	Test Canker	Transgenic Strain	Average Canker Size
	154	untreated	52 mm
	392	untreated	71
	154	146/pXH9	29
	392	155/pXH9	60

Plot 2	Test Canker	Transgenic Strain	Average Canker Size
	154	untreated	46 mm
	392	untreated	57
	154	146/pXH9	53
	392	155/pXH9	51

In 1994, there were a number of ascospores that were transgenic. Instead of the expected 50/50 ratio (non-transgenic/transgenic), only 30% of the spores were transgenic. It is not known if there is a reduction in germination to explain the 70/30 ratio. No transgenic ascospores were recovered

in 1996 or 1997, but mass isolates that are white and hygromycin resistant can still be recovered a year later.

Remote sampling: no transgenic spores were recovered from rain water. Airborne spores that were transgenic were found in the following numbers:

5/35 in October 1995
15/1183 in October 1996

New cankers also were examined. One white, hygromycin sensitive isolate (Tp39) was found below a treated canker. It was a new v-c group. Another isolate (Tp51) was white and hygromycin resistant. It was a unique v-c type and cDNA-derived. It was a superficial canker; it is felt this canker was ascospore initiated.

He and Anagnostakis are looking at population replacement in the test plots in the Meshomasic Forest. They have three indigenous strains representing both mating types and different v-c types; they were transformed with pXH103 = Not1 site in the 3' non-coding region. They are looking at risk assessment in the Meshomasic Forest by examining non-target woody species and insects.

He also reported on transformation of *C. cubensis*, an important pathogen of *Eucalyptus* sp. He is collaborating with Mike Wingfield in South Africa.

Mark Double, West Virginia University

He reported on the evaluation of genetically engineered strains of *C. parasitica* that are currently being field tested. Don Nuss genetically engineered dsRNA from EP 713 into EP 155, (orange) and EP 146 (brown). A hygromycin cassette was included to confer antibiotic resistance. The objectives of this study were to: (1) monitor the ability of the engineered strain to survive and spread from infected bark patches, used as inoculum sources, to pre-established virulent infections; (2) determine whether ascospores and conidia carry virus particles from the engineered strains; and, (3) compare dissemination efficiency of the engineered strains with comparable strains that carries the same but cytoplasmically-borne virus.

Four plots were established in the Monongahela National Forest in July 1994. Within each plot, 16 healthy American chestnut trees were selected for study. Three virulent cankers were established on 8 of the 16 trees; 8 trees were left uninoculated. In October 1994, bark patches inoculated with a transfected or transformed *C. parasitica* strain were placed 10 cm above the virulent cankers. All canker/bark combinations were vegetatively incompatible but sexually compatible. Bark patches were replaced periodically. Pigmentation was used as a marker for both the recipient and donor (bark patch) strains. The treatment combinations were as follows:

Plot	Virulent Canker	Donor Strain
1	EP 146 (brown)	EP 155/pXH9
2	EP 146 (brown)	EP 155 (cytoplasmic)
3	6-7-1 (orange)	EP 146/pXH9
4	6-7-1 (orange)	EP 146 (cytoplasmic)

Cankers were sampled by removing small bark plugs in December 1994, May 1995, November 1995, May 1996 and October 1996. Cultures obtained from the plugs were evaluated for pigmentation, colony morphology, presence and absence of hypovirus, and resistance to hygromycin. Findings for the mass isolates include: (1) with few exceptions, isolates recovered from the artificially established or naturally occurring cankers were representative of the strains used to establish cankers; and, (2) white, hygromycin-resistant colonies were isolated but they were not recovered over time.

Ascospores were examined from perithecia that were collected in October 1996. Ascospore progeny (n=45,000) were analyzed from single perithecia to evaluate recombination with the bark patch source of inoculum. Findings from the ascospore progeny were:

- Results were similar to 1996, but recombination with the engineered strain was reduced.

- No white ascospores were recovered from plot 1 and only 2% of the ascospores from plot 3 were the result of recombination with EP 146/pXH9.
- Approximately 50% of the perithecia from EP 146-initiated cankers (plots 1 and 2) were a result of "selfing". This is in contrast to <18% in the EP 155-initiated cankers (plots 3 and 4). Approximately 77% of the perithecia in plot 3 were a result of outcrossing to wild-type inoculum and 5% were a result of outcrossing with the recombinant isolate from the bark patch.

Spermatization Study. A new study has been initiated to discern the best time for spermatization and the best method of spermatization. Cankers were initiated in July 1997 on 24 trees with 6-7-1, an orange isolate that is sexually compatible with EP 146/pXH9. Hypovirulent inoculum was dispensed via three methods: peptone broth, thickened agar slurry and bark patch. The intent was to spermatize in July, August and September. Permission from APHIS was not given until August; therefore, the dates were changed to August, September and October. Perithecia will be collected and ascospores examined to determine the best method and date of spermatization.

Lou Shain, University of Kentucky

He reported on polygalacturonase (PG). There is some evidence that PG is a virulence factor. Greater amounts of PG are found in EP 155-induced cankers than in hypovirulent-induced cankers.

Chinese chestnut has far more PG inhibition than American chestnut. The next logical step is to disrupt PG and see what kind of canker disruptants are produced.

Strain	Mean Canker Area (cm)
EP 155	12
EP 713	3
Transformants:	
40-2-2	14
192-4	12
40-1-5	12

The above data suggests that PG is not a virulence factor. Acidic PG isoforms have been sent to Nuss for cloning and sequencing.

Hebard experienced dieback in some trees at the Meadowview Farm. Hebard sent dieback material to Shain and the resultant organism was found to be *Coryneum castaneicola*. He has single spore isolates that he provided to Hebard for test inoculations to test pathogenicity. Hebard made the inoculations on September 15, 1997.

James Bier, Michigan State University

He is looking at population genetics at six infected chestnut sites in Michigan with varying degrees of recovery. He isolated dsRNA and determined homology using northern blots. He isolated about 60 virus-containing isolates from the following populations:

	CL	FF	GH	RC	AS	ST	Total
Total # samples	82	57	81	78	6	11	315
# Infected with virus	65	53	66	18	1	1	204

CL=County Line; FF=Frankfort; GH=Grand Haven; RC=Roscommon; AS=Augustat; ST=Stivers

He obtained 3 types of viruses:

GH2	RC1	S16VT
9.8		3 bands, but no weights
3.5	2.8	
0.8	1.6	

S16VT type is found at GH, RC, AS and ST.

On occasion, he can find 5 bands in GH2. Some bands come and go, so those bands were never used for diagnostic purposes. He has tried different media, light intensities, solid versus liquid media and he could find no correlation of the bands that appear and disappear.

He has 12 banding pattern types found in the population of *C. parasitica.* Most are similar to the GH2 pattern. Phylogenetically, Frankfort and County Line are the most related while the Roscommon population is the most distinct, using a cluster analysis and distance matrix.

Yir-Chung Liu, Cornell University

Between 1978-81, hypoviruses were released in two areas of West Virginia (Monongahela National Forest and the George Washington National Forest). Hypoviruses were of the CHV1 and CHV3 type. In 1994, cankers from these two areas were sampled and 364 isolates of *C. parasitica* were obtained. The following table lists some of the hybridization results.

Hybridization of dsRNAs from Released and Recovered Isolates

Released Isolates	CHV1-EP43	CHV3-GH2	SR2
1-4-2 w	+	–	–
EP 43	+	–	–
EP 47	+	–	–
EP 50	+	–	–
EP 60	–	+	–
EP 90	–	+	–
EP 93	–	+	+
EP 102	–	–	+

Recovered Isolates	n	dsRNA+	Probe dsRNA		
			CHV1-EP 43	CHV3-GH2	SR2
Release Area					
Monongahela NF	139	35	0	0	35
Geo. Wash. NF	121	47	0	6	47
Surrounding Area					
Monongahela NF	78	19	0	0	8
Geo. Wash. NF	26	6	0	0	6
Other CHV3's					
D2 (PA)	--	--	--	+	--
Natural Br., KY	18	3	0	1	2

Her objectives are to establish the persistence of released hypoviruses and compare virulence of released and recovered hypoviruses. To do this, plots in West Virginia that were used in dissemination studies in the late 1970s were visited in 1994. Cankers from second-growth chestnut stems were sampled. Several virus-containing isolates were obtained. These isolates were screened by immunoblotting, column purification and northern hybridization. Only 6 of the 350 recovered isolates hybridized to GH2. All the recovered isolates hybridized to the SR2 probe.

Her conclusions are that only 6 CHV3-type dsRNAs were recovered in released areas. CHV1-type dsRNA did not persist over the 15 year period since it was last released.

To address her second objective of virulence of released and recovered hypoviruses, she is screening single conidial isolates that are: virus free; CHV3 only; SR2 type only; CHV3 and SR2 type dsRNAs. Field inoculations also will be conducted.

The question still remains as to how dsRNAs got into the recovered isolates. Was it by vertical transmission or horizontal transmission? She will do DNA fingerprinting and dsRNA sequence analysis to this question

Paul Chaloux, West Virginia University

His project is in conjunction with the above study of Yir-Chung Liu. Strains infected with dsRNA that hybridize to CHV3 and/or SR2 dsRNA were recovered from sites where hypoviruses were introduced 15-20 years ago. Single conidial progeny (scp) infected with CHV3-type and SR2-type, CHV3-only, SR2-only dsRNA's as well as hypovirus-free scp have been developed from the recovered sites. In most instances, little or no morphological abnormalities appear to be associated with infection by the recovered dsRNA's. However, some of the recovered isolates are associated with hypovirulent-appearing cankers. An extensive field experiment using conidial progeny from seven recovered isolates and three isolates used during the original hypovirus release is planned for the fall of 1997.

Michael Milgroom, Cornell University

He is studying the genetics of v-c types in *C. parasitica*. In collaboration with Paolo Cortesi of the University of Milan, approximately 60 of 64 possible v-c types and their genotypes have been identified from Italian isolates. These are defined by six *vic* loci, with two alleles each. All 64 genotypes will be identified soon.

Isolates from a population of chestnuts in Finzel, Maryland were collected and 24 v-c types were paired against 57 testers with known *vic* genotypes. Twenty-one v-c types were found in Maryland that were compatible with the genotype testers. Based on these results, there are at least six polymorphic *vic* loci in this Maryland population.

He is also mapping vic genes to find allele specific markers. To date, one cross between an Italian and a Japanese isolate in which five *vic* genes are segregating has been analyzed. Approximately 200 progeny have been assigned to the 32 v-c types determined by these five *vic* genes. In collaboration with the USDA-FS in Saucier, MS, they are embarking on bulked segregant analysis to find RAPD markers linked to each of these *vic* genes.

Reproductive biology of *C. parasitica* is also an area of study. They have found that selfing rates among natural populations is 20-30%. They wanted to know if isolates that "self" contain both mating type alleles. These alleles are termed idiomorphs because they are highly dissimilar in sequence and in length. They have cloned mating type specific markers and have looked at conidia with DAPI staining to rule our heterokaryons. They have found:

- "Selfing" occurs 20-30% in nature but it is rare in the laboratory.
- Idiomorphs segregate in a fashion that correlates 100% with mating-type phenotype.
- Isolates that self do not contain equimolar copies of both idiomorphs.
- Mating types can segregate among single conidia from a single strain.

Clarissa Balbalian, West Virginia University

She has field tested some of David Huber's (Michigan State University) strains to see if unidirectionality and epistasis occur in the field as it does in the laboratory. The primary objective of her study was to evaluate the effects of two *vic* loci on the transmission of hypoviruses between strains of known vegetative compatibility on living chestnut trees. She used strains that differed at 0 *vic* loci, or that differed at the *vic* 1 locus; the *vic* 2 locus or both the *vic* 1 and *vic* 2 loci. Virulent cankers were artificially established in October 1995 and the cankers were exposed one year later to hypovirulent inoculum delivered via a bark patch mounted above the canker. The bark patch inoculum sources contained either a North American (COLI) or European hypovirus (80-2). A reciprocal was established for each pairing to identify cases of epistasis or unidirectional transmission by inverting the roles of the canker-inciting strain and the hypovirulent strain. The artificially established cankers were sampled in November 1996, February and May 1997 to discern the extent of virus interaction.

When the canker-inducing strain and the hypovirulent strain were homoallelic, hypovirus recovery was consistent in both directions for the COLI virus. Hypovirus transmission was greater in one direction than in its reciprocal when 80-2 was the hypovirus. When *vic* 1 was heteroallelic, hypovirus transmission was unidirectional. Hypovirus recovery was greater when 80-2 was the hypovirus than when COLI was the transmitted hypovirus. Hypovirus was not

recovered when *vic* 2 was heteroallelic, or when both *vic* 1 and *vic* 2 were heteroallelic in either of two different combinations.

When hypovirus was transmitted to the canker inciting strain, generally it did not become established throughout the entire infected thallus. Hypovirus recovery rate was not equal among cankers on a tree. One or two cankers often contributed all or a majority of the hypovirulent isolates recovered. Also, hypovirus recovery often was greater on one tree than on its replicate.

Bill MacDonald, West Virginia University

A stand of 2500 American chestnuts in West Salem, WI, covering 50 acres, is the largest stand of American chestnuts in the U.S. Four cooperating agencies are involved in combating chestnut blight that was discovered at the site in 1987. The agencies include: Wisconsin DNR, West Virginia University, Cornell University and Michigan State University. A hypovirus from County Line, MI (COLI) was introduced into the resident West Salem strain and 186 cankers were treated with this virus between 1992-94. Cankers sampled from multiple locations indicated that only 30% of bark plugs each year contained the hypovirus. Further, hypovirus infection was recovered from less than 10% of samples from untreated cankers, indicating limited spread of the hypovirus. These data prompted the introduction of a second hypovirus, Euro 7, into the resident Wisconsin strain. Cankers were treated with this hypovirus in 1995–97. Findings as of 1997 include:

- 132 trees are now infected with 662 cankers; 215 new infections were discovered in 1997.
- The Euro 7 hypovirus has become the most commonly identified hypovirus. In 1997, when *C. parasitica* was recovered, Euro 7 was associated with 38% of the isolates from bark samples removed from previously treated cankers.
- Other organisms (especially *Trichoderma*) were commonly recovered from infections at rates from 11-40%, with older cankers generally yielding a greater number of contaminants.
- A third disease focus, caused by a third vegetative compatibility type, has been discovered in another area of the stand.

OBJECTIVE 3. Continue efforts toward developing blight-resistant chestnuts utilizing both tissue culture and traditional breeding methods.

Phillip Gordon, New York Botanical Garden

He showed a leaf of *Kalenchoe*, a fleshy dicotyledon. It had small plantlets on the leaf, an example of apomyxis. The 3rd and 4th pair of leaves begin to produce new plantlets. *C. dentata* propagates in the exact way but with different parts of the plant. *In vitro* plant tissue culture in the petri dish is apomyxis in nature. When American chestnut is stressed, it sprouts from the base; the same system occurs in corn when it sends out suckers. Chestnut sprouts from the root collar with the same red color as occurs in *Kalenchoe*. It acts as a protectant.

Robert Bernatzky, University of Massachusetts

He is trying to identify regions of the genome of American chestnut that confer host resistance. He is expanding his genetic map of leaf-derived cDNA sequences through RFLP analysis. He has added 7 markers this year, two of which show linkage to loci in the previous set of 16 markers. Five of the new markers do not show linkage to other cDNAs, but linkage relationships with previously mapped isozymes or RAPDs are still to be determined.

What does he intend to do with the markers? Fred Hebard sent some material from advanced breeding lines, primarily based on disease resistance. The markers will be used to come up with an index of how close we are moving (and how quickly) toward American chestnut in the backcross program. He may be able to identify markers that are associated with superior trees. He will collaborate with Tom Kubisiak on a populations genetics study.

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

He is working on two projects:

1. He is mapping the genomic regions that condition resistance to *C. parasitica* in 2 different backcross lines ('Mahogany' and 'Nanking') to verify if these 3 regions are heritable. He wants more Chinese chestnut markers; he would like bands that are present in Chinese and 'Mahogany' and absent in American chestnut. In order to identify markers useful for linkage analyses, he screened a battery of 10-mer primers against DNAs extracted from 'Mahogany' Chinese chestnut and two different bulks of American chestnut recurrent parents. He detected a total of 158 Chinese chestnut-specific RAPD markers. Single-marker regression analyses performed on average canker size in August identified a total of 36 markers that were significantly associated with a resistance response. These 36 markers mapped to 3 different linkage groups. Twenty-two of these markers were located on linkage group C, and are most likely linked to *Cbr1*, one of the resistance loci identified in the previous mapped F_2 population. The most significantly associated marker on linkage group C explained as much as 29% of the observed phenotypic variation Two other markers explained as much as 30% and 18%, respectively, of the observed variation. These results are very encouraging as they confirm previous results that suggest that three unlinked regions are conferring resistance in 'Mahogany' Chinese chestnut.

Results of screening suggest that approximately 80–90% of the markers identified in 'Mahogany' are also present in 'Nanking' and should be useful for linkage analyses.

2. He is looking at levels and structuring of genetic variation in American chestnut. The main goals of this project are to assess overall levels of genetic diversity in wild populations of American chestnut, partition the genetic variation into within and between population components, determine whether there is any geographical pattern to the observed variation, and to develop conservation and breeding strategies.

This program is in its infancy. He hopes to sample across the entire range of American chestnut according to a 135-mile grid. He hopes to have 25 sites located within the natural range in addition to 3 sites in the vicinity of Meadowview, VA. Sixty trees per location will be randomly sampled within a 20 mile radius, with a minimum of 100 meters between trees. Tissues will be subjected to RFLP and RAPD analyses for allelic richness and evaluation of genetic diversity and geographic variation. A total of 10 RFLP loci and 20 RAPD loci will be surveyed.

Fred Hebard, The American Chestnut Foundation, Meadowview Research Farm

He is following the traditional backcross method to get to 15/16th American by the third backcross. Whether this will work depends on the number of genes involved. Resistance is not controlled by many genes, but rather is inherited fairly simply as indicated by the recovery of highly blight-resistant progeny from F_2 generations at frequencies of 5%. It was feared that poor form is linked to resistance, but Hebard has recovered highly blight-resistant trees that appear to have good form.

Three sources of resistance ('Mahogany', 'Clapper' and 'Nanking') are being advanced into 20 lines each of American chestnut from the Meadowview area. He will start production of third backcross F_3 nuts from 'Mahogany' and 'Clapper' about 8 years from now. All lines will be producing about 13 years from now. Attempts are being made to extend the effort with these sources of resistance to other areas of the country. A particularly vigorous effort has started in PA. Currently, seven macroscopic traits are being used to select for American type: leaf hairs, vein hairs, twig hairs, stipules, bud shape, stem color, time of bud break and male sterility. Molecular traits (in conjunction with USDA-FS, Saucier, MS) are being developed.

Hebard is using markers to select trees that have high number of American traits that are not linked to resistance, via RAPD markers.

Scott Schlarbaum, University of Tennessee

He set out variety trials of grafted clones and seedlings in 1994 in Tennessee. Two of the trials had sufficient mortality that they will be abandoned. Several trials are marginal. He has potted chestnut for a bee trial using four varieties of bees as pollinators.

James Hill Craddock, University of Tennessee at Chattanooga

He has five cultivar evaluation sites. They have experienced terrible mortality; most have succumbed to Ambrosia beetles. This may be associated with sun scald.

He reported that gall wasp has been found at the McMinnville, TN site, an area well known for its nursery stock. He subsequently transferred his germplasm collection from McMinnville to Knoxville. He has cultivars that are European, European x Japanese and Chinese. He found that trees grew best in pots treated with Spin-Out, a copper-containing root pruning compound.

He reported a putative Phytophthora problem at a chestnut orchard in the Natchez region of Mississippi.

He is working with a local area land trusts and he hopes to develop a site on campus at the University of Tennessee at Chattanooga.

Robert Doudrick, USDA-Forest Service, Southern Institute of Forest Genetics

He has hired a post-doc to work on the cytogenetics of pine and do a complete genome analysis of gymnosperms and angiosperms. There has been talk of including American chestnut in this study. Chinquapin material has been collected already.

Business Meeting

Dennis Fulbright was elected secretary for 1998-99. Chairman for 1997-98 is Don Nuss. Next year's meeting will be held in Maryland at a site to be chosen by Don Nuss. The meeting will be September 10–12, 1998.

The agenda for the business meeting was as follows:

- common nomenclature
- name of fungus
- project renewal

Common Nomenclature. Anagnostakis said that a standardized system needs to be published for the Ascomycetes. Gilleon Turgeon published a convention of plant pathogenic genes where:

a = mat1
A = mat2

This is based on the concept of idiomorphs, mat1-1 and mat1-2. This has been abandoned in most cases; it is now mat1 (a) and mat2 (A). It follows the *Neurospora* system.

The gene product is always CAPS.

Name of Fungus. Anagnostakis recapped that *Endothia parasitica* was changed to *Cryphonectria parasitica* by Margaret Barr-Bigelow, who looked at type cultures and published a monograph of the *Diaporthales*, based mainly on ascospore septation. Nuss commented that there is not much sequence information on other species using rRNA. He felt a careful study should be done using current molecular tools. This would enhance our stand with common nomenclature.

Jarosz commented that the term, chestnut blight fungus, is not a good name, since the fungus also affects oaks. Anagnostakis responded by saying that chestnut blight fungus is the official name of the organism. van Alfen commented that we must make sure the proper name is used with respect to the audience He also suggested that when referring to an isolate, uppercase 'EP' should be used for consistency.

Hebard disagreed with the use of healing and non-healing or lethal and non-lethal cankers. He felt that reactive and non-reactive cankers is better terminology. It was suggested that the following may be the most descriptive:

non-reactive (lethal) and reactive (non-lethal)

Doudrick responded by saying that phenotype is not always reflective of what is going on inside the canker.

Viral Nomenclature. MacDonald questioned how to correctly report Euro 7 with and without virus. Hillman reported that an update of the Hypoviridae will come out in the 7th edition of Virus Taxonomy and Nomenclature. He said the main consideratons in virus taxonomy are:

1. Most viruses that we are working with are in the family *Hypoviridae*, genus *Hypovirus*.
2. All *C. parasitica* viruses are not alike, such as the mitochondrial viruses. Those are not members of the genus Hypovirus, nor are viruses in isolates such as 9-B-2-1 or C-18. Hillman said in the case of Euro 7, it is clearly a CHV1-type virus, so call it: CHV1-Euro 7. The fungal isolate would simply be Euro 7. For the fungal name, if the Euro 7 virus is in another fungal background, the correct nomenclature would be: EP 155[CHV1-Euro 7].

Bertrand mentioned that the *Neursopora* system is very easy to work with, due to its nomenclature. He cautioned that it will be very confusing if the group discontinues using 'EP' as a prefix to most isolates. Hillman agreed that since official viral names are as follows: CHV1-EP 713, then the prefix, 'EP' must continue to be used. He continued by saying that all *C. parasitica* isolates are not always prefixed with EP, such as NB58 and Euro 7.

Hillman suggested that since Euro 7 has always been a virus-containing isolate, rather than using Euro 7 (v) as a virus-free isolate, we should use a single conidial number to refer to a virus-free Euro 7. He also suggested that a short disclaimer on each publication would be in order to cover viruses that are not characterized yet.

MacDonald questioned how we cite double infections. Hillman responded that double infections would appear as: EP 155 [CHV1-Euro 7, CHV3-GH2]. A comma would separate the two viruses within the brackets.

Bertrand said a mechanism should be in place to handle nomenclature questions. He felt there should be an *ad hoc* committee to make recommendations as needed. Anagnostakis will field questions about fungal nomenclature and Hillman will respond to questions on viral nomenclature.

Project Renewal. van Alfen commented that Anagnostakis should be commended for her efforts to produce the draft report. He reported that three reviewers sent back comments on the proposed renewal. The committee is obligated to respond to the reviewer's questions in a response section. Anderson informed the group that of the 3 reviewers 2 were with the USDA-FS and one was with an Agriculture Experiment Station.

Anagnostakis said she obtained information from members and how each intends to cooperate via their specific projects. She looked at what projects we currently have and how they compare to the 'national objectives'. She figured we could address 3 of the 'national objectives', so she invented 3 objectives to go with the national priorities.

Anderson said the committee of Northeast Directors will get the revised version, not the working copy with responses to the criticism. He does not think the document should be drastically rewritten.

There was some discussion regarding the rewrite of the proposal and van Alfen advised the committee to focus on the document and not on the process of how it is rewritten. Anderson then said that the Directors will look at one term, interdependency, and pass or reject the proposal based solely on this one term. He commented that regional research is not comprised of individual activities but regional research is based on interdependency of one group to another. The primary test for approval will be, "Is there sufficient evidence for interdependency"? The Northeast Directors do not want individual work. The guidelines used by the Directors are:

- necessity of sharing costly equipment
- need to adequately test material in diverse regions
- collaboration on databases
- complex research project

MacDonald questioned whether the focus of the proposal should be on chestnut or as a "model system". Hebard felt that both should be a focal point. van Alfen said the NE-140 project has had a leading effort as a model system project. Anagnostakis said the focus of the national guidelines has changed over the years and model systems are not a priority. The national focus now is slanted toward feeding the world and agriculture. Bertrand believes the Northeast Directors are concerned about the science of the project. Anagnostakis asked how many Directors are scientists and Anderson reported that most are administrators. Huddle reported that the government performance act is driving many administrators and they have to justify the money they spend. The key point is probably not model systems but more grass roots.

van Alfen felt that things can be wrapped together in a framework of a science approach and tied into the things we are tying to accomplish. Doudrick felt that there are not enough people in NE-140 to accomplish new objective 3. We do not have any land managers and that may be a bit shortsighted. Hebard said that the American Chestnut Foundation hopes to have trees sold on a commercial scale in 10 years and land managers are not needed yet. Schlarbaum suggested that we should begin establishing American chestnut stands in forests now. This would lend us a working knowldege of how to manage chestnut stands so we are prepared for the backcross trees that will available in the future. He proposes that we grow 4-5' seedlings to find dry ridge sites where chestnut can be established. Doudrick then proposed that an additional paragraph may be needed that mentions the important role of models that is dovetailed into a sentence that incorporates land managers, so the breeding objective does not stand alone.

van Alfen said he would like a document that keeps the group unified, regardless of whether the Northeast Directors accept or reject the proposal. Hillman agreed and voted for expediency with regard to the proposal as long as it encompasses what we intend to do. Craddock felt the word interdependency should appear in the first paragraph of the proposal. MacDonald agreed to take the justification section and work on it.

Discussion then revolved around incorporating objective 3 into objective 1 or leaving it as a separate objective. Schlarbaum commented that we are moving closer to beginning to restore chestnut in the forests so we should begin working on understanding chestnut in the forest. He feels we can rewrite some things into the objectives where we currently have expertise. He agreed to "beef up" objective 3 but incorporate it into objective 1.

It was agreed that any wording changes or inserts should be sent to Anagnostakis by October 5, 1997. She will send the national priorities to MacDonald and he will rewrite part of the justification. She urged everyone to look critically at the description of cooperators and send any changes to her.

The meeting was adjourned at 10:30 am on September 20, 1997.