NE-140 Technical Committee Meeting
Biological Improvement of Chestnut (*Castanea* spp.)
Cayuga Nature Center, Ithaca, NY
24-25 September, 1993

Attendance:

Connecticut: Sandra Anagnostakis, Philip Gordon
Georgia: Daniel Carraway
Kentucky: Lou Shain
Massachusetts: Terry Tattar, Mark Mount
Michigan: Dennis Fulbright
New Jersey: Bradley Hillman, James Polashock, Ping Wang
New York: Michael Milgroom, William Powell, Yir-Chung Liu, Robert Marra, Susanne Lipari, Martin Bissegger, Yang Zhou, Tobin Peever, Kerong Wang, Alice Churchill, Bill Powell, Stan Wirsig
Texas: Neal Van Alfen
Virginia: Fred Hebard
West Virginia: William MacDonald, Mark Double, Scott Enebak
Ontario: Colin and Beatrice McKeen

The meeting was called to order at 8:30 am September 24, 1993 by Chairman Milgroom. John Czamanske, caretaker at the Cayuga Nature Center gave a welcome address. The nature center is a private, non-profit education center that was built in the 1920's by the WPA. It has 120 acres, complete with trails, fitness trail and nature walks.

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

Michael Milgroom, Cornell University

Much of the organization of the meeting was done by Susanne Lipari. No USDA representative was in attendance. Dick Rohde, Administrative Advisor, cancelled at the last minute due to a death in the family. NE-140 has been approved for another 5-years, until 1998.

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

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

She reported that she is testing a number of recombinants made by Don Nuss at Roche Institute in the greenhouse, using woody stems. The strains of *C. parasitica* , with a cDNA copy of the hypovirulence genome of strain Ep 713 in the nucleus of strain Ep 155, were tested on twelve species of woody plants growing in pots. The various woody stems were inoculated with Ep 155 and the recombinant strain CN2 in 4-mm-diameter holes. Lesion length for Ep 155 were greatest in American chestnut (63 mm) and red maple (32 mm), while lesion length for CN2 were greatest in sweet birch (12 mm) and red maple, sugar maple and red oak (10 mm). An application is in progress to APHIS to release strain CN2 in two field sites in West Virginia (W. MacDonald) and Connecticut (S. Anaganostakis) in collaboration with Don Nuss.

Michael Milgroom raised the question as to the fitness of the agent from Ep 713. Neal Van Alfen felt we are entering a new frontier; his only concern was the time-frame may be longer than anticipated. Fred Hebard questioned whether the spread can be successfully monitored.

Neal Van Alfen, Texas A&M University

His laboratory is investigating the function of Vir1 and Vir2 genes, using Ep 155/2, UEP1 (an isogenic form of Ep 155/2 containing Ep 713 dsRNA) and dm18 (a deletion mutant from Ep 155/2 that has the Vir2 gene deleted). The characteristics of Vir1 and Vir2 genes are as follows:

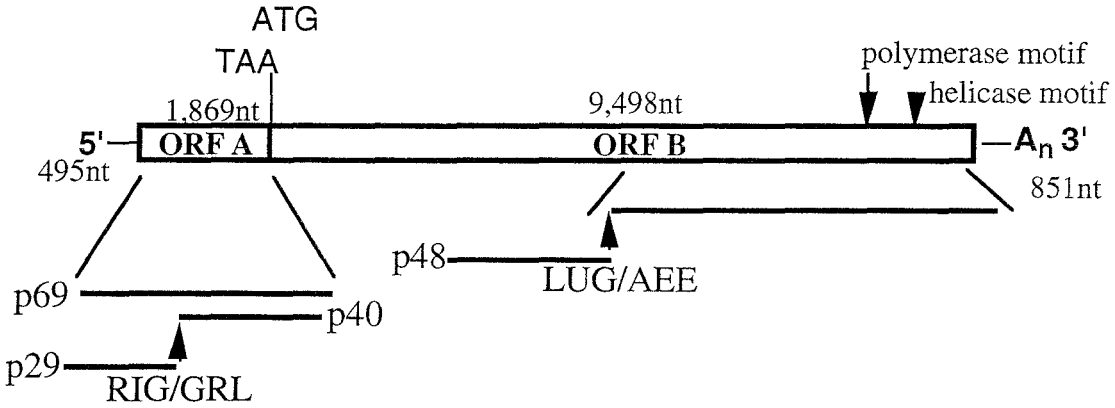
- the Vir2 gene deletion mutant dm18 has reduced sporulation
- in an attempt to cross dm18 and Ep 144, perithecia were produced that were barren
- Vir1 and Vir2 are mating-type specific A, not a
- Vir1 and Vir2 contain no introns--they are transcribed as mRNA and there are numerous open reading frames--the ORF1 is identical in both Vir1 and Vir2, the terminal sequence is CAAX
- the time courses of mRNA expression
- dsRNA suppresses Vir2

He has found that a propheromone is encoded for. The amino acid sequence of propheromones are identical for *C. parasitica* and other fungi, such as *Ustilago maydis, Saccharomyces cerevisiae, S. phombe, Cryptococcus neoformans.* In yeast propheromones, the mating-type locus regulates pheromone production in A cells. There is a receptor on the α cells, and in turn a transcriptional activator activates cell fusion, nuclear fusion, cell cycle arrest, pheromone production and secretion. Mating-type genes are primarily cell regulators, an implication for *C. parasitica.* He believes that mating type needs to be looked at, as it may be controlled by dsRNA.

He followed up on the recombinant made by Don Nuss and tested by S. Anagnostakis. This Ep 713 transformant may be suppressing sexual reproduction and limiting the evolution of the fungus. He believes conidia that contain dsRNA may be sexually sterile and only those without dsRNA are fertile.

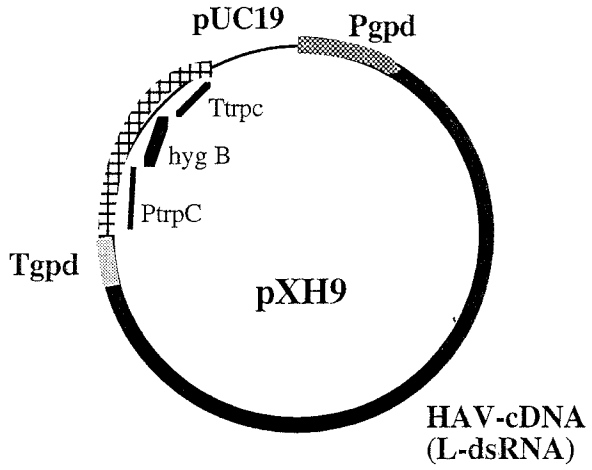
Ping Wang, Roche Institute of Molecular Biology

He showed the following schematic of L-dsRNA of Ep 713 that was used by Baoshan Chen, Gil Choi and Donald Nuss in their paper, "Mitotic stability and nuclear inheritance of intergrated viral cDNA in engineered hypovirulent strains of the chestnut blight fungus.



He reported the four transformants were produced, CN2, CN3, CN6, and CN7. CN2 was chosen as the transformant that may be used in release studies (see Anagnostakis, page 1). Chen, et al. reported stable maintenance of integrated viral cDNA through repeated rounds of asexual sporulation and passages into host tissue. The transformation plasmid, pXh9, used in their study to generate the engineered hv strains, contains a full-length cDNA copy of the viral L-dsRNA present in Ep 713, under the control of the *C. parasitica* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd-1*) promoter and terminator, and the *Escherichia coli* hygromycin B

phosphotransferase gene (*hygB*) flanked by the transcriptional contol elements of the *Aspergillus nidulans typC* gene, as shown below:



They demonstrated stable nuclear inheritance of the integrated viral cDNA and resurrection of the cytoplasmic viral dsRNA form in progeny resulting from the mating of an engineered hypovirulent *C. parasitica* strain and a vegetatively incompatible virulent strain. Mitotic stability of the viral cDNA ensures highly efficient transmission of the hypovirulence phenotype through conidia. Meiotic transmission, a mode not observed for natural hypovirulent strains, introduces virus into ascospore progeny representing a spectrum of vegetative compatibility groups, thereby circumventing barriers to ananstomosis-mediated transmission imposed by the fungal vegetative incompatibility system.

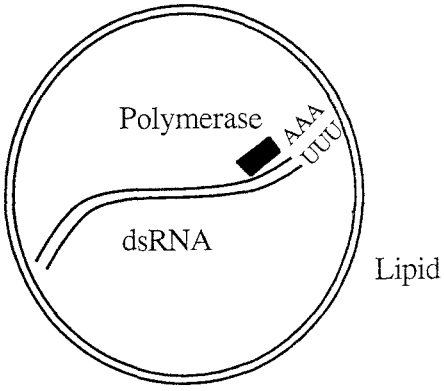
The paper by M. Craven, D. Pawlyk, G. Choi and D. Nuss, entitled "Papain-like protease p29 as a symptom determinant encoded by a hypovirulence-associated virus of the chestnut blight fungus", reports viral dsRNA responsible for virulent attenuation also profoundly influences a range of host functions in addition to virulence. Also suppressed are fungal sporulation, pigmentation, and accumulation of the enzyme laccase. They mapped this suppressive activity to the autocatalytic papain-like protease, p29, present within the amino-terminal portion of open reading frame A-encoded polyprotein p69. They found that p29 is necessary but not sufficient for the level of virus-mediated suppression of fungal pigmentation, sporulation, and laccase accumulation observed for wild-type hypovirulent strain Ep 713 and is nonessential for viral RNA replication and virulence attenuation.

Brad Hillman, Rutgers University

He described the proposal to the ICTV to name the virus associated with Ep 713 and NB58. The proposal was accepted at the Glasgow meeting, and the taxonomic structure of the family is as follows:

Family: *Hypoviridae*
Genus: *Hypovirus*

The taxonomic committee consisted of Bradley Hillman (chair), Dennis Fulbright, Donald Nuss and Neal Van Alfen. The virus can be sited as follows: CHV1-713 or CHV2-NB58. Questions were posed as to the use of the nomenclature and Hillman responded that we are making up the rules as we go along. He said that we are trying to name viruses and the strain descriptors will probably hold up. This is the first virus accepted by ICTV that has no capsid.



Some of his salient points were:

- phylogenetically this virus is related to plant potyviruses and dsRNA is excess of 10 kb will probably be in this genus, *Hypovirus*
- developing the taxonomy will be time-consuming
- use in the literature will determine its acceptance

He indicated references to use to cite CHV are the 6th annual ICTV report. He is also submitting the nomenclature to Arch. Virology.

Laboratory updates are as follows:

1. C-18 and 9B-2-1 characterization. He has switched from C-18 to 9B-2-1, since the latter has a distinct morphology associated with it, unlike C-18. He has made a cDNA library of 9B-2-1 and now has 700 clones. If there was conservation between C-18 and 9B-2-1, it would be with the larger segments. To date, clones for 2 segments have been mapped and there is no cross-hybridization of large segments between C-18 and 9B-2-1.

2. NB58 results. The genetic organization is like Ep 713; there is no proteolytic cleavage site. What is the nature of leader on translation *in vivo*? If GUS fusions of the leader are made in yeast, GUS is shut down. In transformed *C. parasitica*, whether of not you have the leader, GUS production is high. There appears to be something in *C. parasitica* that circumvents shutting down GUS with the leader.

3. Isolates collected by Peter Bedker. Ninety percent of these isolates are virulent and 10% hv. All the NJ isolates look alike on petri plates. They examined the dsRNA and amplified various pieces: near the 5' end and predicted little conservation; in the middle and predicted moderate conservation; and, a larger piece and predicted high conservation. Results indicated they needed the highly conserved region. Every one of the hv isolates had at least one base change; none were identical in this conserved region, and some differed as much as 10%. NB58, whether kept on a lab bench, in the refrigerator, or on a tree; the sequence is identical--there is no drift. Even on adjacent trees, isolates are not identical.

4. Strain 747, Matt Brown's work. Ep 747 differs from Ep 713. It is less stable and gives more flat mutants. He characterized the genome and the bottom line is there is no indication that Ep 747 is fundamentally different from Ep 713. Its proteolytic site is identical but he does not know about the cleavage site. He has yet to determine what gives rise to the variation between Ep 747 and Ep 713.

5. Mating results with NB58.

Mating	**Progeny Phenotype**	**% Conversion**
Ep 155/58-19	58-19 (Ep 155)	35
Ep 155/58-F	58-F	0
Ep 155/58-F	Ep 155	33

They examined 300 ascospores and found that morphology seems to go with the virus. NB58-F is avirulent and segregates as a nuclear gene.

James Polashock, Rutgers University

He examined a very small autonomous dsRNA from *C. parasitica*. His findings are as follows:

- it is a 2.7 kbp dsRNA element
- it has no effect on culture morphology (the isolate with this element is NB632)
- he made a cDNA library and the whole virus expressed a single full-length transcript
- there is no reduction in sporulation
- in apple studies, NB632 and Ep 713 were grew significantly less than Ep 155
- since it is not morphologically different, it must be screened either for dsRNA or on plated out on Bavendamm's medium
- it is transmitted through 100% of the conidia and 50% of the ascospores
- it is transmitted cytoplamsically
- there is no long ORF
- the only long ORF is in the mitochondria, leading them to believe this element may be closely associated with mitochondria
- when running a phylogenetic tree, NB632 is more closely related to the yeast T & W elements
- it has been shown to be double-stranded RNA

Dennis Fulbright, Michigan State University

He reported on several areas of research.

1. dsRNA-free hv isolates. He examined a dsRNA-free isolate from the Kellogg Forest. He looked at the phenomenon of single conidia and he subcultured various parts of the petri plate culture and often, within 2 subcultures, the culture was dead. He went back to the bark to see if this senescence phenomenon was laboratory related and senescence was found also in freshly isolated cultures. This is a cytoplasmically transmissible to other strains. Single conidial isolates were examined and nuclear RFLPs were the same as the mother culture; mtDNA RFPLs were different than the mother culture. Mitochondrial plasmids are present, but there is no correlation to virulence.

2. He is continuing to use respiratory inhibitors to look at normal and alternative respiration. Strain Ep 155 has little alternative oxidation, while CL125 has 75% of its oxidation via an alternative pathway. Ethidium bromide/UV light mutagenesis of Ep 155 has yielded constitutive products of alternative oxidase. Some of the mutants are hypovirulent. He questions if heteroplasmons are being created.

3. mtDNA. Physical mapping of mtDNA of Ep 155 has shown 6 restriction enzymes (80 kb complete for 4 enzymes our of 130 kb total). A large rDNA has been located, cob. They will be doing small rDNA, coI, ndh, and others.

4. V-C conversion. When loci are identical, transmission is 100%. When loci differ by one locus, transmission is lower, but it still occurs. One gene can block dsRNA transmission 100% of the time, while others will allow 50% transmission. "Liners" can be found through nuclear transfer.

Neal Van Alfen, Texas A&M University

He reported on two areas of research, down-regulation of genes and virus replication.

1. Genes down-regulated in CHV1-133 infected strains are:
   - MatA pheromones (Vir1 and Vir2)
   - cell surface hydrophobin, cryparin (has 50% homology with *Certocystis ulmi*)

- extracellular laccases (LacA, LacC)
- intracellular laccases (LacB)
- cutinase

In the hypovirulent strain, UEP1, Vir1 and Vir2 are not expressed, nor is laccase expressed, howver, cryparin is expressed. Regarding nuclei isolation, he raised the question as to how the above genes are regulated. One way to look at this is to look at pathways: are they down-regulated at the transcriptional level? Laccase, Vir1, Vir2 and Cry are all down-regulated by comparing virulent and hypovirulent levels by mRNA expression.

2. Virus replication. Vesicles were loaded onto a gel and electrophoresed. A DNA complex is formed on the gel that can be removed by RNAse in low salt. How many proteins are produced and which ones are coded for? He took the complex and separated it by a gradient and probed for proteins. Some proteins are unique to the complex and not found in the rest of the strain. A 87kd protein is probably the RNA-dependent RNA polymerase.

Alice Churchill, Boyce-Thompson Institute

She isolated genes by functional complimentation; she compared two mutant strains:

Mutant Strain 1	**Mutant Strain 2**
-methionine auxotroph	-methionine auxotroph
-normal pigmentation	-reduced pigmentation
-asexual sporulation reduced 1-20% of WT	-asexual sporulation reduced 0.001% of WT

She constructed a total genomic DNA library and transformed into mutant strain 2. This resulted in 7 transformants and she rescued the resulting cosmid. The insert in the cosmid was 39 kb. She locallized the gene in the 39 kb piece and got it down to a 3.3 kb fragment. The 3.3 kb fragment, when inserted into the auxotroph, would compliment the mutant to produce more pigmentation and sporulate more heavily. She questioned whether integration was at a homologous site and a non-homologous site. Her results indicated homologous integration. The size of the transcript is approximately 3.0 kb.

She has tried to isolate the PIG gene. This required screening thousands of transformants. The media she was using did not allow her to identify any transformants. She changed culture media that allowed pigment production and she was able to rescue a cosmid from 1 transformant. She is in the process of characterizing the cosmid.

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

Phil Gordon, New York Botanical Garden

His interest has been in survival mechanisms of organisms, wounding and healing. He comes from a background in the pharmaceutical industry, namely antibiotics. He is interested in immune mechanisms and he believes that plants possess an immune system, or have an equivalent system. He became familiar with tissue culture to acquaint himself with wounding and this all leads to survival mechanisms. In 1988, Sandy Anagnostakis and Charles Burnham convinced him to look at the disease process in American chestnut. It is his best estimation that root collar sprouting is somatic embryogenesis. He is looking at chestnuts in the forests of Connecticut and he has found chestnut everywhere. He want to piggy-back onto the American Chestnut Foundation's breeding program, so when a resistant tree is developed he can cross this hybrid with old, flowering trees in Connecticut to produce nuts and adapt them to local environments.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

1. Rocky Hill site. Rocky Hill is an old woodlot (~1 acre) that was never clearcut. It contains abundant chestnut. She began using hv is 1982 and she finished treating in 1986. She compared Rocky Hill trees with other Connecticut plots, without hv introduction, where data has been taken for many years. She looked at basal area in square centimeters per sprout clump and compared Rocky Hill and 4 other long-term areas. Rocky Hill averaged 90 cm^2. while the other plots averaged ~ 10 cm^2. Another comparison is the change in size class distribution between 1977-87. The trees at Rocky Hill are larger than in the long-term forest plots, leading to the conclusion that hv is a factor.

2. Chemistry-peroxidase activity. Peroxidase activity is 4 times higher in Chinese vs American chestnut bark. Tissue prints using a nylon-blotting technique showed that there is more peroxidase activity at the margins of cankers than in healthy bark. Since peroxidase enzymes use Mg^{++} as a cofactor, bark of both trees was examined for amount of several minerals, and for distribution of Mg^{++} and other ions. Using an ICP to analyze ground, extracted bark samples, she found 2 times more Mg^{++} in Chinese chestnut bark than in American chestnut bark in April-cut stems with a peak just behind the canker that was 6 times the base level. The only other mineral with increased amounts around the canker was phosphorous.

Fred Hebard, The American Chestnut Foundation, Meadowview Research Farm

He used the bark-wood assay with Chinese and American chestnut, and he found significantly smaller cankers on Chinese wood, than on American or first hybrid wood. He used a virulent isolate, SG2-3, which is a virulent isolate with reduced pathogenicity. He wanted an isolate with low pathogenicity to see if he could distinguish between American and first hybrids; SG2-3 does not discern first hybrids from American chestnut.

Bill MacDonald, West Virginia University

He reported on the American chestnut trees at West Salem, Wisconsin. As of July 1993, *C. parasitica* isolates from 74 cankers have been tested for their v-c type, by pairing on PDA. To date, only one v-c type has been found. This was confirmed by Michael Milgroom, who did DNA fingerprinting and all isolates examined yielded the same DNA fingerprint. During the winter of 1991-92, one isolate from the Wisconsin collection (Wisc 25-1) was successfully converted to the hv phenotype by pairing, in dormant chestnut bark, with a hv isolate from County Line, Michigan (COLI 11-1). This converted West Salem isolate (Wisc 25-1/COLI 11-1) has consistently converted (*in vitro*) isolates from 74 cankers at the West Salem site. In June 1992, all known cankers within 10 meters of the ground were treated with the Wisc 25-1/COLI 11-1 isolate. A subsequent treatment was made in 1993 that included all cankers that developed after those treated in 1992. In July 1993, a limited number of samples were removed from areas where visible stroma had formed beyond the treatment areas. Of the 11 cankers sampled, 7 yielded isolates that exhibited hv morphology and contained dsRNA, indicating successful transfer of the dsRNA. In addition, 5 previously untreated cankers were sampled; 2 were dsRNA positive and had hv morphology, indicating some natural spread of hypovirulence.

Scott Enebak, West Virginia University

He evaluated the biological control potential of two uniquely different dsRNA viruses discovered in West Virginia, C-18 and 9B-2-1. These viruses are structurally different than those associated with typical hv isolates, and qualify as true viruses as they contain a protein coat. Results have shown that C-18 converted 3 of 37 v-c groups, while isolate 9B-2-1 did not convert any of the 37 v-c groups. The ability of these particular isolates to transmit their dsRNA to other isolates may be as much a function of the genetic background of the host isolate as the infecting dsRNA. This hypothesis came about because C-18 dsRNA, when transmitted into a brown,

virulent isolate, Ep 146, was able to convert 8 of 37 v-c groups. In dissemination studies, C-18 appears to have the potential to pass the virus into conidia (31/500) and ascospres (6/500) more effectively than the isolate 9-B-2-1 (21/500 conidia, 0/750 ascospores). No 9B-2-1 ascospores have yet been found that carry the virus. Experiments to determine whether C-18 and 9B-2-1 can inhibit canker growth are underway.

Mark Double, West Virginia University

He reported on the cooperative experiment with Gary Griffin of Virginia Tech, in which cleared and non-cleared plots are compared to discern disease incidence and movement of hv isolates. In the spring of 1988, plots were established in a 5-year old clearcut with abundant chestnut regeneration. Of the 12 plots established, 6 were cleared of competing vegetation; the remaining 6 were not cleared. Four European hv isolates were inoculated in 3 cleared and 3 non-cleared plots by scratch-wounding 25% of the trees. New infections were sampled and isolate morphology examined twice a year, from 1988-93. New cankers yielding isolates with hv morphology from hv-inoculated plots have remained somewhat constant, but disappointingly low (~10%). Fifty-three percent of the hv isolates have been recovered from trees that received hv inoculum. Dissemination is currently limited due to high tree mortality (>80%).

Terry Tattar, University of Massachusetts

Investigations are underway to attempt to locate naturally occurring hv strains of *C. parasitica* in Massachusetts forests. A major point of focus of this study is the Quabbin Watershed in western Mass; it has stands with a low density of American chestnut (< 5 trees/acre) and low incidence of blight. The Metropolitan District Commission of Massachusetts wants to increase biodiversity of this stand, and attempts will be made to decrease the *C. parasitica* population, via mud-packs or the introduction of hv isolates.

Colin McKeen, Canadian Chestnut Council, Ontario, Canada

He prefaced his remarks by stating that many researchers are impatient with hv and its rate of spread. He encouraged patience. He stated that there are two different types of hv in Ontario, the Arner tree type (dsRNA-free) and the typical dsRNA type. There are 7-8 sites that harbor the conventional dsRNA hypovirulence; these have discrete, healing cankers.

He has followed the Arner tree (source of non-dsRNA-containing hypovirulence) from 1967 to 1973 and then again from 1984 to the present. This tree came from an old stump. It had a 3" sprout that died in 1983, so the fungus is virulent. He and Greg Boland (University of Guelph) have sample 15 times around the circumference to determine if the fungus is consistent throughout the canker. He does not have these results yet. He cannot see cankers far up in the tree, as it is now ~75' tall. There is a smaller tree nearby the Arner tree that has large swollen cankers. This tree contains dsRNA, confirmed by Boland and Dennis Fulbright. Trees near Niagara Falls have been treated with mud-packs. These cankers exhibited break-over, so he has inoculated them with the Arner isolate. He has had some success and some failure with the Arner isolate

At some sites, he has used a "cocktail" mix of;' this contains the Arner isolate and 3 dsRNA-containing isolates. This mix is moderately pathogenic as it will kill small stems.

There are 15-18 trees across southern Ontario that are < 22 " dbh.

Michael Milgroom, Cornell University

The following individuals are working on chestnut blight in his laboratory:

-Susanne Lipari-technician
-Yang Zhou-technician
-Yir-Chung Liu-graduate student
-Robert Marra-graduate student
-Tobin Peever-post-doctoral fellow
-Kerong Wang-visiting scientist from Nanjing Agricultural Center, China

-Martin Bissegger-visiting scientist from Switzerland

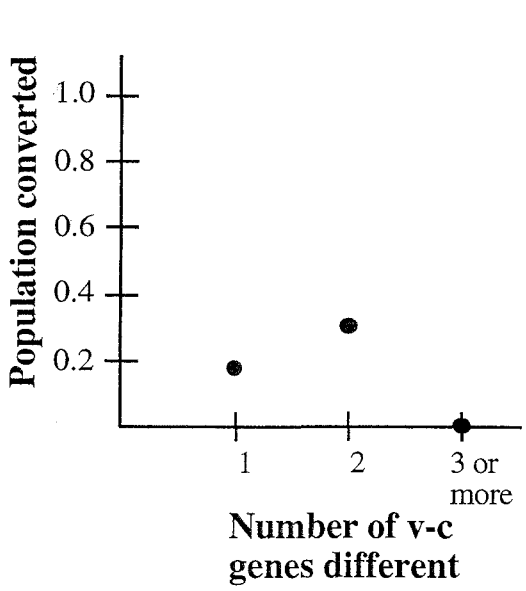
Yir-Chung Liu, Cornell University

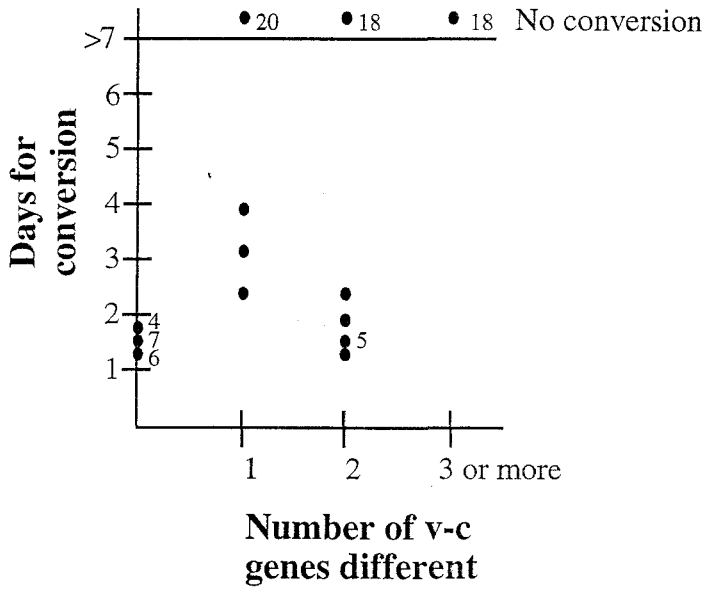
She reported on hypovirulence studies within and among isolates in different v-c groups. Her hypothesis is the rate of transmission of hv is correlated to the number of v-c genes different among isolates. Her sample population was from Finzel, Maryland and v-c typing was done by Mark Double for 58 isolates. To compare the relatedness, she did transmission tests by:

- randomly selected 8 isolates of different v-c groups
- converted 8 isolates by hv strains, Ep 43 and Ep 113
- she compared conversion tests for all Maryland isolates by pairing with the 8 hv isolates (she compared the morphology of samples and virulent strains over time to determine the number of days for conversion)

Crosses have been made among a subset of the isolates to determine the number of *vic* genes different between isolates. Progeny were paired with each parent to determine the number of parental v-c types. The following are preliminary results from 85 crosses that have been made to date. When virulent isolates were converted, it occurred within 2-4 days. All pairs of isolates in the same v-c group were converted successfully. No pairs with 3 or more vic genes different showed conversion. Conversion between isolates with 1 or 2 vic genes different occurred in 5 of 24 and 8 of 26 pairings, respectively. These results, which confirm earlier studies, provide quantitative estimates of transmission rates in relation to v-c group relatedness at the population level. No attempts have been made to determine the effects of individual genes on transmission.

# Genes different	Parental :	Non-parental
1	1 :	0
2	1 :	1
3 or more	1 :	3+





Regarding DNA fingerprinting, she will compare fingerprints and v-c types, excluding the non-reactive isolates. At this point, she has 38 isolates to compare. Her results, to date, show that DNA fingerprint diversity within v-c groups is slightly smaller than diversity among v-c groups.

Michael Milgroom, Cornell University

He discussed the need to model the genetics of the chestnut blight fungus. His laboratory is looking at the population genetics of the fungus. He raised the question regarding population subdivision, are we looking at one large population or distance smaller populations? To answer this question, he is looking at several distinctly different *C. parasitica* sites:

Depot Hill, NY
Danby, NY
Mt. Lake, VA
Finzel Swamp, MD
Concord, NH
Frankford, MI

He has examined 15-70 isolates from each population and extracted DNA and constructed RFPLs at 6 unlinked loci. He has found that allele frequency are different (some significant differences among populations). To analyze gene diversity, he has used the following equation:

$$\hat{}H = 1 - \sum p_i^2$$

$$H_T = H_S + D_{ST}$$

where H_T is the total population division, H_s is the within population division and D_{ST} is between population division. Therefore,

Gene diversity = $G_{ST} = D_{ST}/H_T$

Population	G_{st}
All populations	0.34
All populations - Michigan	0.16
Michigan	0.81

His conclusion is *C. parasitica* populations in the eastern U.S. are moderately subdivided and there may be restricted gene flow.

He sampled a single *C. parasitica* isolate from every chestnut blight canker in 3 forest plots of approximately 30 x 30 meters in NY to look at spatial autocorrelation of genotypes. A sample from a much larger plot in Finzel, MD (250 x 100 meters) was collected from 55 cankers on trees with stems larger than 2.5 cm. Three different types of molecular genetic markers were used: DNA fingerprinting; single copy RFPLs; and, mitochondrial DNA. His results are:

- DNA fingerprinting genotypes aggregated in 3 small plots due to restricted movement on a local scale
- When identical genotypes in the same chestnut stem were removed, there was still more aggregation than could be explained by chance
- mtDNA haplotypes were significantly aggregated in one plot, but not in another
- There were too few isolates with the same fingerprint genotypes to do this analysis for the MD population
- Genetic differences determined from DNA fingerprinting were significantly correlated to Euclidean distances between pairs of isolates in the 3 small plots, but the association was not strong

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

Bill Powell, Syracuse University

He is collaborating with Chuck Maynard and they are looking at any organism with resistance genes, such as the edible snail, frog skin, *Xenopus*, and moths. He is looking at small peptides from organisms that may be antifungal. He is hoping to sequence one small peptide 20-

24 amino acids in length. He is interested in looking at different promoters to try to regulate the expression in plants.

Fred Hebard, The American Chestnut Foundation, Meadowview Research Farm

He is breeding to dilute out all Chinese characteristics except blight resistance. Backcrossing is not practical if more than 3 genes are involved.

He commented that *C. parasitica* isolates on Chinese chestnuts are irregular, rather than elliptical. In Japanese chestnut, football-shaped callus is formed. The fungus nearly totally colonizes the vascular cambium, although this does not lead to tree death; this may be similar to butt swell of Scarlet oak.

He inoculated 200 F_2 (1/2 Chinese) and 400 B_1F_2 (3/4 Chinese). He plants his blocks in a totally randomized design with American and Chinese chestnut interplanted. To test virulence, he inoculated 6" above the ground line with Ep 155 and further up the stem with SG2-3 (his less aggressive virulent isolate). He sees fairly small cankers on the Nanking cultivar. Seedling Chinese chestnut are less resistant than Nanking or Meiling; there is extreme susceptibility in his F_2 population. If there are only a few genes controlling resistance, there should be a wide variation in susceptibility/resistance, and this is indeed the case. He feels his data shows that there are probably 2 genes responsible for blight resistance. He is certain that it is not 1 or 4 genes. He therefore believes that the backcross method will be successful.

Using trees with low, intermediate and high level of blight resistance, his canker growth statistics indicate that Floyd cannot be distinguished from Chinese chestnut, using slope of mm of canker growth/day. Floyd and Chines can be distinguished from American using this measurement. Using canker length (mm) he can distinguish Chinese chestnut from American chestnut and first hybrids, but he cannot use canker length to distinguish American chestnut from first hybrids.

Daniel Carraway, University of Georgia

His approach to resistance is from the side of the host plant. He believes that a combination of hv and a resistant host will be the best way to develop a timber-type tree. He is trying to develop a gene transfer system via somatic embryogenesis (*in vitro* regeneration system). Somatic cells go through maturation, and his laboratory has been working with various tree species, such as Yellow poplar, Magnolia and oak. He has X-plants from immature embryos from Wisconsin, New York, Connecticut, Pennsylvania, North Carolina and Georgia.

He is working on two projects simulaneously and hoping to bring them both together:

- a regeneration protocol
- a gene transfer protocol

1. Regeneration protocol. Growth regulators are used to produce and proliferate early globular stages and then are removed to allow the developmental stages to occur. He is trying to find the optimum zygotic stage to use as the embryo and he has found that the early stages work best. Embryos go though various stages, such as early globular stage to heart stage to torpedo stage. His problem has been getting root maturation before shoot maturation. The embryos have not developed enough to form shoots prior to root maturation begins. Therefore, he is trying to regulate growth so that root and shoots develop at a similar time. He has experienced some conversion, but not enought to make the system work. He is now using different osmotica and looking at ABA to get the system to a point where he can ge sufficient numbers of entire plants.

2. Gene transfer protocol. He needs cultures of globular cells that are synchronous. He uses "gene gun" system, whereby he bombards plated material with gold microprojectiles that are coated with plasmid DNA, that encodes a selectable marker. The DNAS is attached to a carrier that is forced into the cells via high-pressure helium. He assays for transient GUS expression 2 days following bombardment. He then transfers his bombarded material for selection and he looks for kanamycin resistant colonies. He uses the following substrates in his analysis:

GUS Assay	**Substrate**
Histochemical	X-Gluc

Fluorometric	MUG
Spectophotometric	p-NPG

He has the gene transfer protocol worked out. He now need to select a gene that will be useful (antifungal properties). His problem is the regeneration of plants. He can occaisonally get entire plants, but that is rare. He is continuing to work on the regeneration problem while searching for using a useful gene to insert.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

She established a relationship with Russian scientiştst. They have photographs that show chestnut blight that kills young shoots, in the former republic of Georgia.

Crosses were made this past year to get chinquapin into various chestnut lines. The rationale is that *C. pumila* is not susceptible to gall wasp (*Dryocosmus kuriphilus*). Since gall wasp has been reported on the Appalachian trail in northern Georgia, it will most likely move north. All crosses were made on Rocky Hill American chestnut seedlings in an orchard in Connecticut, with various *C. pumila* cultivars as male parents.

She sent American chestnut seed to Jerry Payne at the Fruit and Nut Tree Station in Byron, GA. Jerry planted the seed and then tested for susceptibility to gall wasp and he found that American chestnut is as susceptible as Chinese chestnut. Sandra has warned growers in northern Georgia and sourthern Tennessee to check their nursery stock prior to shipping, as the chance for dissemination is great via nursery stock. Scott Schlarbaum has found gall wasp in Asheville, NC at the Bent Creek demonstration forest. Jerry Payne believes *C. pumila* is resistant to gall wasp, but he does not know the resistance of *C. ozarkensis*.

Lou Shain, University of Kentucky

He is looking at antifungal hydrolases in terms of host resistance. He is specifically looking at two hydrolases: chitinase and β-1-3 glucanase. These two enzymes were induced in both American and Chinese chestnut in response to fungal challenge or incubation with the plant stress hormone, ethylene. The mechanism of these two enzymes appears to be oligosaccharides that are released and serve as ellicitors of antifungal compounds. Acidic and basic isoforms of both hydrolases were detected in native protein from both species on activity gels after isoelectric focusing and polyacrylamide gel electrophoresis using the Davis or Reisfeld buffer system for acidic-neutral or basic proteins, respectively. Basic chitinase is located in vacuoles while acidic chitinase is secreted out of intercellular spaces. Protein extracts from Chinese chestnut bark inhibited *C. parasitica* more than extracts from similar amounts of American chestnut bark. Protein from Chinese bark was fractionated by anion-exchange chromatography. Bioassays revealed that antifungal activity resided in the basic fraction.

A single extracellular endopolygalacturonase was induced in culture filtrates of *C. parasitica* strain Ep 155 with 1% sodium polypectate. This enzyme is implicated in several plant pathogen systems. The enzyme was purified via several steps (ultrafiltration, cation exchange and gel filtration on Sephadex G-75) and its molecular weight was approximetaely 42 kDa. The K_m and V_{max} were calculated to be 0.22 mg/ml and 0.241 mmol, respectively. The purified enzyme induced browing of the inner bark of chestnut.

Business Meeting

Brad Hillman was elected secretary for 1994. Chairman-elect for 1994 is Scott Merkle; the 1994 meeting will be hosted in Athens, GA. M. Milgroom will write the 1993 termination report.

Bill MacDonald reported on the status of the proceedings of the International Chestnut Conference held in Morgantown, July 1992. He projected the cost to be between $7,000-9,000. While many problems have been encountered, the publication should be completed by December 1993.

Dick Rohde sent along the following information:

- $2.5 million has been moved from IPM to competitive grants for biological control

• the USDA deadline for competitive grants is December 6, 1993

The meeting was adjourned at 3:00 pm on Saturday, September 25, 1993.