NE-1033 2009 Annual Report

<u>OBJECTIVE 1</u>. To develop and evaluate blight resistant chestnut trees for food and fiber through traditional and molecular techniques that incorporate knowledge of the chestnut genome

William MacDonald, West Virginia University

Backcross orchard for assessment of host resistance combined with hypovirulence W.L. MacDonald and M.L. Double (in cooperation with Fred Hebard, Bob Paris and Sara Fitzsimmons, The American Chestnut Foundation). Six replicate plots containing 150 trees each were established at the Plant and Soil Sciences Farm in Morgantown, WV in the spring of 2005 for the purpose of assessing the interaction of host resistance and virulent/hypovirulent strains of *Cryphonectria parasitica*. Seeds have been replanted annually from 2006-2009. Despite a continued effort to water, fertilize, mow and control weeds, only 60% of the planted seedlings survived as of August 2009.

Fred Hebard, The American Chestnut Foundation

Meadowview Update (Fred Hebard, Bob Paris and William White). Meadowview experienced drought conditions in 2007 and into 2008. However, there was enough rain during the 2007 growing season to make most crops, except hay, which suffered from drought in May and early June, when production peaks. The drought did not decrease chestnut production significantly, but did impair growth of new seedlings on some soils.

Inventory. Our current holdings are presented in Table 1, and changes from 2007 to 2008 are indicated in Table 2. We now have more than 47,000 trees and planted nuts, an increase of almost 14,00 over last year (Table 2). Most of the increase is due to the addition of B_3-F_2 trees, which increased by 13,071. Our holdings of other nut types are relatively constant, with plantings of those offset by removal, as we have made selections and rogued the rejects. However, the degree of backcrossing is increasing; for instance, we added 410 straight B_4s in 2008 and two new lines. Reclassification of some crosses also affected some statistics, for instance, when Chinese chestnut trees were reclassified as Chinese x Chinese trees if they were products of controlled crosses.

We were very fortunate this year to start planting B_3s from the 'Nanking' source of blight resistance at the Virginia Department of Forestry's Matthews State Forest in Grayson County, Virginia, which is adjacent to Meadowview's Washington County. We had needed an isolated location for 'Nanking' B_3s in order to harvest B_3-F_2s relatively free from pollen contamination. We plan currently to convert this breeding orchard in place into a seedling seed orchard. The planting at the Matthews was made possible by Ed Stoots, Wayne Bowman and Zack Olinger of the Virginia Department of Forestry, and their colleagues.

Harvest. The most exciting news is that we harvested 1,883 B_3-F_3 nuts in 2007, a ten-fold increase from the previous two years. Hopefully, the number of harvested B_3-F_3 nuts will exceed 20,000 in two or three more years! Some of these B_3-F_3 nuts were sown in forest nurseries in the winter of 2007-2008 to be set out into forest test plantings in 2009 in cooperation with the U.S. Forest Service. So the light at the end of the tunnel TACF has been in for 25 years is coming into view. Here's hoping these B_3-F_3 fare well. In addition to National Forest plantings, B_3-F_3 nuts and seedlings also are being distributed to members, prioritized by seniority of membership.

The year 2007 saw our largest harvest ever, almost doubling last year's harvest. The main factor increasing the harvest was that almost all the straight B_3s from Clapper and Graves have now been screened for blight resistance and are approaching full production; we harvested 31,220 B_3-F_2 nuts from them.

In addition, we harvested almost 3,795 nuts from the new "Father Tree Program" where chapters bring American chestnut pollen to Meadowview to make crosses. This is especially advantageous $_{for}$ the southern chapters, where their American chestnut trees flower early, often before

anthesis in Meadowview, necessitating use of last year's pollen. Additionally, due to Phytophthora root rot, many of their flowering American chestnut trees are on steep slopes and inaccessible to bucket trucks.

Finally, it was a good year for nut production in the Meadowview area, and many trees bore well. An exception was Chinese chestnut, which suffered from a hard freeze over Easter weekend of 2007, after starting to break bud due to warm weather in March. American chestnut, and most backcross trees were not affected by the freeze because they had not yet started to break bud.

***Blight resistance screening in B_3-F_2 seedlings*.** The year 2007 was the fourth in which we screened 'Clapper' B_3-F_2 seedlings for blight resistance and the third for 'Graves' B_3-F_2 seedlings. The results of the 'Clapper' seedlings are presented in Table 4 and those for the 'Graves' in Table 5. Unlike last year, there were no significant differences between Clapper lines in blight-resistance, but significant differences occurred between 'Graves' lines, as they had last year.

The resistance ratings were better for 'Graves' than 'Clapper' B_3-F_2s. but this may reflect better growing conditions at the farm with the 'Graves' than the farm with the 'Clapper' seedlings. This is the second round of chestnuts planting at the 'Graves' farm, our original Wagner Research Farm, and chestnuts do better on these old agricultural soils in the second than the first round of planting.

We have now completed planting a fair proportion of our 'Clapper' B_3-F_2 lines, where planting of 1350 nuts marks completion. In 2008, we had completed planting 12 lines of Clapper B_3-F_2s and had started 27. For Graves, 4 lines are completely planted and 20 started. The rate of planting between 2002 and 2007 for Clapper lines is shown in Figure 1. Usually it has taken about 4 years to complete the breeding of those lines which are complete, ranging from 1 to 7.

We would like to thank Lou Silveri, Dave Lazor, and Sam Fisher for helping with pollinations and inoculations. Special thanks to Dave Slack for volunteering two days a week all year round for the past three years! Also, we need to acknowledge the role of George Sykes,Danny Honaker, Darryl Caudell, Lori Hall, Louise Cottrell and many others in keeping the farms running from day to day. Thanks to all —this wouldn't get done without their help. If you are interested in helping to pollinate next year, plan on any time in June (call 276 944-4631). If you are interested in learning more about the Elder Hostel program, call 617 426-8055 or write 75 Federal St., Boston MA 02110.

Table 1. Type and number of chestnut trees and planted nuts at TACF Meadowview Research Farms in May 2008, with the number of sources of blight resistance and the number of American chestnut lines in the breeding stock.

Type of Tree	Number of		
	Nuts or Trees	Sources of Resistance	American Lines*
American	2006		210
Chinese	562	54	
Chinese x American: F_1	475	21	79
American x (Chinese x American): B_1	522	14	29
American x [American x (Chinese x American)]: B_2	1744	11	43
American x {American x [American x (Chinese x American)]}: B_3	1796	9	81
Am x (Am x {Am x [Am x (Ch x Am)]}):B_4	440	3	5
(Ch x Am) x (Ch x Am): F_2	317	5	7
[Ch x Am) x (Ch x Am)] x [Ch x Am) x (Ch x Am)]:F_3	5	1	1
[Am x (Ch x Am)] x [Am x (Ch x Am)]: B_1-F_2	471	4	4
{Am x [Am x (Ch x Am)]} x {Am x [Am x (Ch x Am)]}:B_2-F_2	240	6	6
(Am x {Am x [Am x (Ch x Am)]}) x (Am x {Am x [Am x (Ch x Am)]}):B_3-F_2	31240	2	47
B_3-F_3	96	1	2
Chinese x (Chinese x American): Chinese B_1	184	3	4

Chinese x [American x (Chinese x American)]	41	1	1
Chinese x {American x [American x (Chinese x American)]}	435	5	16
Chinese x Chinese	2463	70	
Chinese x Japanese	109	2	
Chinese x European	140	1	
Chinese x Large, Surviving American	288	10	10
European	1	1	1
European x American F_1	2	1	1
Japanese	10	4	4
Japanese x American F_1	9	2	2
[(Japanese x American) x American] B_1	10	2	2
{[(Japanese x American) x American] x American} B_2	134	2	2
Japanese x European	157		
Japanese x Large, Surviving American	27		5
Castanea seguinii	48	3	3
Large Surviving American F_1	785	19	47
Large Surviving American B_1	446	8	31
Large Surviving American B_2	94	2	6
Large Surviving American I_1	1508	21	23
Large Surviving American I_2	364	6	6
Large Surviving American F_2	150	6	10
Large Surviving American other	64	6	6
Other	31		
Total	**47414**		

* The number of lines varied depending on the source of resistance. We will have to make additional crosses in some lines to achieve the desired number of progeny per generation within a line. In keeping with past practice, the number of lines for each source of resistance are added separately; thus, progeny from two sources of resistance that share an American parent would be counted as two lines rather than one line (this only occurs rarely).

Table 2. Changes between 2007 and 2008 in the number of chestnut trees and planted nuts of different types at TACF Meadowview Research Farms, including changes in the number of sources of blight resistance and the number of American chestnut lines in the breeding stock.

Type of Tree	Increase or Decrease* in Number of		
	Nuts or Trees	Sources of Resistance	American Lines
American	-155		-12
Chinese	-587	1	
Chinese x American: F_1	-36	1	-4
American x (Chinese x American): B_1	-60	-2	-11
American x [American x (Chinese x American)]: B_2	61	0	-52
American x {American x [American x (Chinese x American)]}: B_3	113	0	3
Am x (Am x {Am x [Am x (Ch x Am)]}):B_4	410	0	2
(Ch x Am) x (Ch x Am): F_2	64	0	-2
[Ch x Am) x (Ch x Am)] x [Ch x Am) x (Ch x Am)]:F_3	-1	0	0
[Am x (Ch x Am)] x [Am x (Ch x Am)]: B_1-F_2	0	0	-2
{Am x [Am x (Ch x Am)]} x {Am x [Am x (Ch x Am)]}:B_2-F_2	17	1	-1
(Am x {Am x [Am x (Ch x Am)]}) x (Am x {Am x [Am x (Ch x Am)]}):B_3-F_2	13071	0	12
B_3-F_3	-121	0	-3
Chinese x (Chinese x American): Chinese B_1	-7	0	1
Chinese x [American x (Chinese x American)]	0	0	0
Chinese x {American x [American x (Chinese x American)]}	435	5	16
Chinese x Chinese	208		
Chinese x Japanese	0		
Chinese x European	0		
Chinese x Large, Surviving American	260		
European	0	0	0
European x American F_1	0	0	0
Japanese	-3	1	1
Japanese x American F_1	-2	0	0
[(Japanese x American) x American] B_1	0	0	0
{[(Japanese x American) x American] x American} B_2	1	0	0
Japanese x European	0		
Japanese x Large, Surviving American	-15		
Castanea seguinii	0	0	0
Large Surviving American F_1	237	4	15
Large Surviving American B_1	-85	0	4
Large Surviving American B_2	0	0	0
Large Surviving American I_1	97	2	2
Large Surviving American I_2	364	6	6
Large Surviving American F_2	-224	1	0
Large Surviving American other	-82	-4	-7
Other	-155		-12
Total	**13958**		

* The decreases in Chinese, F_1, B_3, and Large, Surviving American trees reflects roguing of trees with inadequate levels of blight resistance. The increases reflect further breeding and collecting.

Table 3. The American Chestnut Foundation Meadowview Farms 2007 nut harvest from controlled pollinations and selected open pollinations.

Nut Type*	Female Parent	Pollen Parent	Pollinated			Unpollinated Checks			Number of American Chestnut Lines**
			nuts	bags	burs	nuts	bags	burs	
B_1	American	F_1 mollissima12	21	69	137	0	7	20	1
B_1	F_1 mollissima10	American	0	10	21	0	0	0	1
B_1	F_1 mollissima7	American	7	62	124	0	12	16	1
B_1-F_3	B_1-F_2 Clapper;Graves	open pollinated	4351		2419				10
B_2	American	B_1 72-211	15	81	201	3	8	24	1
B_2	B_1 72-211	American	26	65	82	0	4	5	1
B_2	B_1 MusickChinese	American	118	110	264	0	13	28	3
B_2	B_1 Nanking	American	538	635	1762	3	51	142	9
B_2-F_2	B_2 Nanking	B_2 Nanking	19	76	227	0	8	18	1
B_2-F_3	B_2-F_2 Mahogany	open pollinated	655		405				1
B_2-F_3	B_2-F_2 opClapper	open pollinated	2446		1471				2
B_3	American	B_2 Douglas	255	89	141	1	10	19	4
B_3	American	B_2 Meiling	113	98	134	0	8	16	1
B_3	American	B_2 Nanking	598	260	655	8	30	71	9
B_3	American	B_2 R11T14	14	44	120	1	4	6	3
B_3	B_2 Meiling	American	2	10	10	0	2	0	1
B_3	B_2 Nanking	American	441	157	400	0	14	51	6
B_3	B_2 R11T14	American	207	180	539	0	19	73	2
B_3-F_2	B_3 Clapper	open pollinated	19663		12456				50
B_3-F_2	B_3 Graves	B_3 Graves	90	117	255	1	10	29	3
B_3-F_2	B_3 Graves	open pollinated	11467		8699				37
B_3-F_3	B_3-F_2 Clapper	open pollinated	1883		1062				10
B_4	American	B_3 Douglas	16	76	140	0	7	14	2
B_4	American	B_3 R11T14	185	41	141	0	5	22	1
B_4	American	B_3 R1T7	489	309	799	11	34	88	17
B_4	B_3 R11T14	American	6	41	74	0	4	6	2
B_4	B_3 R1T7	American	192	54	129	0	5	10	2
F_1	American	Chinese Meiling	56	36	60	0	4	11	1
F_1	American	Chinese Nanking	110	166	338	0	15	39	8
F_1	American	Chinese Vanuxem	84	65	157	0	7	11	4
Jap B_2	Japanese B_1 PI#104016	American	10	8	9	0	1	1	1
LSA B_1	American	LSA F_1 NCChamp	421	87	239	0	9	21	1
LSA B_1	Irradiated F_1 NCF179	American	91	97	363	1	12	33	1
LSA B_1	LSA F_1 Corrigan	American	340	118	210	0	11	16	3
LSA B_1	LSA F_1 NCChamp	American	55	68	69	0	7	8	2
LSA B_1	LSA F_2 DaresBeach	American	5	3	6	1	1	4	1
LSA B_1	LSA F_2 Ort	American	1063	312	739	1	20	60	4
LSA B_1	LSA I_1-F_1 opWeekly	American	21	11	13	0	1	1	1
LSA B_2	LSA B_1 Corrigan	American		3	6	0	1	1	1
LSA F_1	American	LSA I_1 SciCliffs;Gault	510	124	257	0	9	31	1
LSA F_1	LSA B_1 DaresBeach	LSA F_1 DaresBeach	28	23	74	0	2	5	1
LSA F_1	LSA op CareyMacon2	American	7	11	13	0	1	1	1
LSA F_2	LSA F_1 NCChamp	LSA F_1 NCChamp	8	164	166	0	15	15	1
LSA I_1	LSA F_1 DaresBeach	LSA B_1 DaresBeach	60	25	51	0	2	3	1
LSA I_1	LSA F_1 NCChamp	LSA F_1 Amherst	80	114	237	0	14	27	1
LSA I_1	LSA F_1 NCChamp	LSA op WayahBig	0	33	28	0	4	8	1
LSA I_1	LSA F_1 Ort	LSA F_1 NCChamp	30	26	33	0	1	3	1

LSA I_1	LSA F_1 Ort	LSA op WayahBig	62	146	140	8	11	12	1
LSA I_1	LSA I_1 SciCliffs;Gault	LSA F_1 Amherst	111	41	118	0	4	12	1
LSA I_1	LSA op WayahBig	LSA F_1 NCChamp	38	33	71	0	3	3	1
LSA I_2	LSA F_1 Amherst	LSA I_1 SciCliffs;Gault	14	25	43	0	2	1	1
LSA I_2	LSA I_1-F_1 opDaresBeach	LSA I_1 SciCliffs;Gault	47	12	28	0	2	3	1
LSA I_2	LSA I_1-F_1 opWeekly	LSA I_1 SciCliffs;Gault	383	91	216	4	7	16	1
B_3&B_4	chapter		3795	2605	6488	48	250	784	38
	Parent 1	**Parent 2**							
CxC	Fifteen Chinese	Meiling Chinese	492	232	510	0	27	56	
CxC	Twelve Chinese	Nanking Chinese	494	156	343	0	18	30	
CxC	Eight Chinese	Vanuxem Chinese	72	74	132	0	10	16	
B_3xC	Seven B_3s	Meiling Chinese	150	194	484	0	18	36	7
B_3xC	Seven B_3s	Nanking Chinese	546	184	543	2	22	51	7
B_3xC	Seven B_3s	Vanuxem Chinese	173	172	443	1	20	50	7
LSAxC	Three LSAs	Meiling Chinese	37	75	124	0	5	16	3
LSAxC	Seven LSAs	Nanking Chinese	287	132	264	2	14	32	7
LSAxC	Two LSAs	Vanuxem Chinese	27	43	93	0	4	11	2
B_3-I_2	Clapper B_3	Graves B_3	53	31	46	0	5	9	1
other			119	84	143	17	0	6	
Total Controlled Pollinations, w/o Chapter			**9461**	**5951**	**13563**	**48**	**589**	**1375**	

*LSA denotes Large, Surviving American, being an American chestnut over 13 inches in diameter at breast height (54 inches) that has blight but has survived longer than approximately 10 years.

**The number of American lines for this table is restricted to the number of American chestnut trees that were direct parents, not grandparents, of progeny.

Table 4. Number of 'Clapper' B_3-F_2 seedlings ranked in various blight resistance classes in 2007.

Susceptible Great Grandparent	LS Mean Resistance Rating**		Standard Deviation of Resistance Rating	Number of Progeny Tested	Blight Resistance Class*		
					3	4	5
QBA1CL	4.1	A	0.7	281	91	110	80
QBF3CL	4.1	A	0.7	30	9	12	9
RCF1C	4.2	A	0.7	39	11	12	16
LFR4T14	4.2	A	0.7	87	23	26	38
LFR4T12	4.2	A	0.8	42	13	8	21
HBW1C	4.2	A	0.8	60	20	8	32
HBW3C	4.3	A	0.7	15	3	5	7

* Trees were only inoculated with a weak, but virulent strain of the blight fungus in early June. A rating of 3 indicates that the cankers were small, about 1-cm long, 5 months after inoculation. A rating of 4 indicates the cankers were slightly larger, 2-4 cm long, and a rating of 5 indicates the cankers were over 5 cm long.

** Means followed by the same letters are not significantly different at $p<.0.05$ by a Tukey-Kramer HSD test.

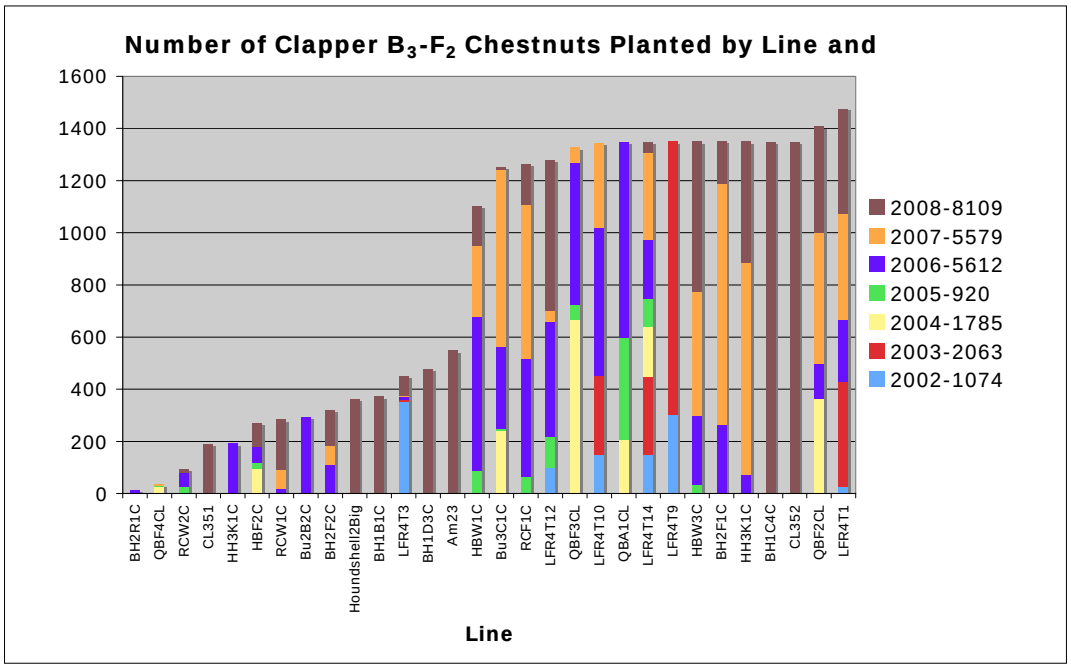
Table 5. Number of 'Graves' B_3-F_2 seedlings ranked in various blight resistance classes in 2007.

Susceptible Great Grandparent	LS Mean Resistance Rating**		Standard Deviation of Resistance Rating	Number of Progeny Tested	Blight Resistance Class*		
					3	4	5
Bu3C3C	3.8	B	0.8	304	149	85	70
Hesper-McGreg	3.8	AB	0.5	19	5	13	1
RCF5GR	4.1	AB	0.8	9	3	3	3
PaulGalloway	4.1	A	0.7	69	19	29	21

* & ** See footnotes to Table 4.

A Quick Guide to Chestnut Breeding Terminology

Parents		Offspring
American x Chinese	=	F_1, "F-one"
F_1 x F_1	=	F_2, F-two
F_2 x F_2	=	F_3, F-three
F_1 x American	=	B_1, first backcross, or B-one
B_1 x American	=	B_2, second backcross, or B-two
B_2 x American	=	B_3, third backcross, or B-three
B_3 x American	=	B_4, fourth backcross, or B-four
B_1 x B_1	=	B_1-F_2, B-one F-two
B_1-F_2 x B_1-F_2	=	B_1-F_3, B-one F-three
B_2 x B_2	=	B_2-F_2, B-two F-two
B_2-F_2 x B_2-F_2	=	B_2-F_3, B-two F-three
B_3 x B_3	=	B_3-F_2, B-three F-two
B_3-F_2 x B_3-F_2	=	B_3-F_3, B-three F-three



Adam Dale, University of Guelph, Canada

Chestnut breeding in Ontario. The Canadian Chestnut Council started a breeding program for blight resistance in 2001. For the first six years we have been supported by a grant from the Government of Ontario's Trillium Foundation and, for the last two, by donations, a few small grants and lots of volunteers.

We have three goals:

- To breed a blight resistant Canadian population of American chestnut using resistance derived from Connecticut Agricultural Experiment Station hybrids.
- To breed blight resistant American chestnuts using surviving trees found in Ontario.
- To maintain clones of surviving American chestnuts in Ontario so that the existing genetic diversity in the Province is not lost.

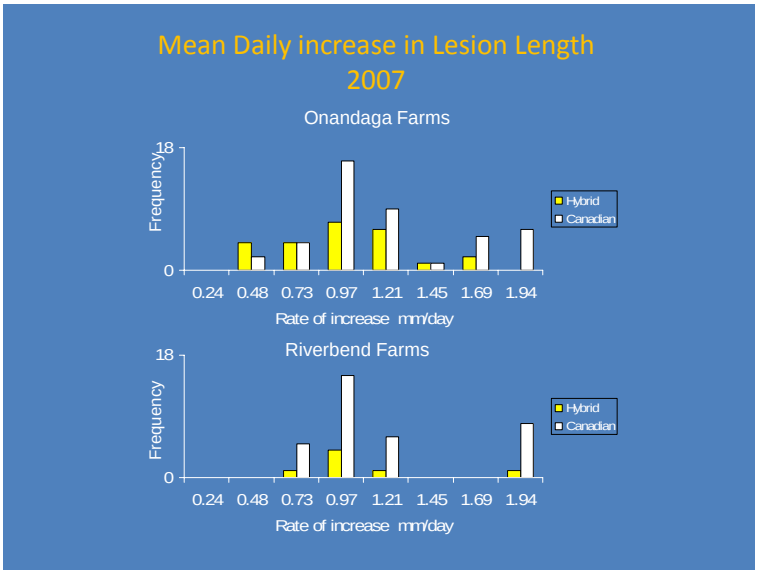
To date, we have a population of 636 trees hybrid which are hybrids between 14 Ontario trees as females and three of Dr. Sandra Anagnostakis' trees as males, and 805 Canadian trees, either from controlled crosses or open-pollinated seeds. These have been planted at two sites, Tim Horton Foundation, Onanadaga Farm, St George ON and Riverbend Farms, Aylmer, ON.

We have been inoculating trees for the last three years. We have the largest population of American chestnut germplasm in Canada, with at the best, only moderate resistance to chestnut blight. Also, we wish to maintain this germplasm for other traits and legally we cannot kill the trees. So, we are using branch inoculations with two isolates on side branches in each of two years for each tree.

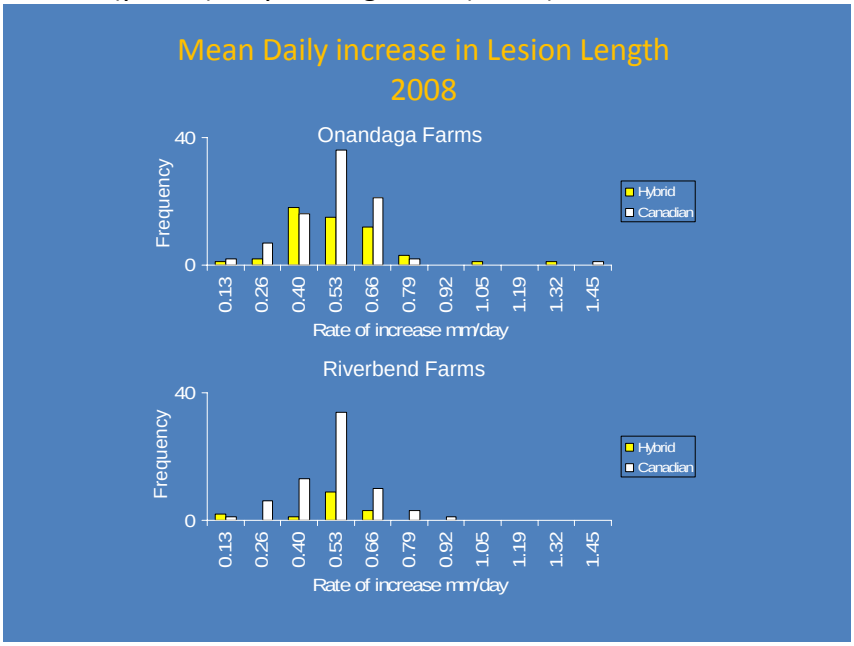
The results from 2007 and 2008 indicate that both the hybrids and the Canadian trees showed a similar normal distribution of lesion lengths at both sites. This has led us to conclude that there may be usable blight resistance in our Canadian population. Four hybrid and six Canadian trees have been identified as potential candidates for the next round of hybridizations and these were started this year.

Rooted American chestnut cuttings. Cuttings are the cheapest and most efficient way to propagate vegetatively, and we often lose grafts because the union breaks or becomes infected. Consequently we decide to try to develop a way to root American chestnuts cuttings directly.

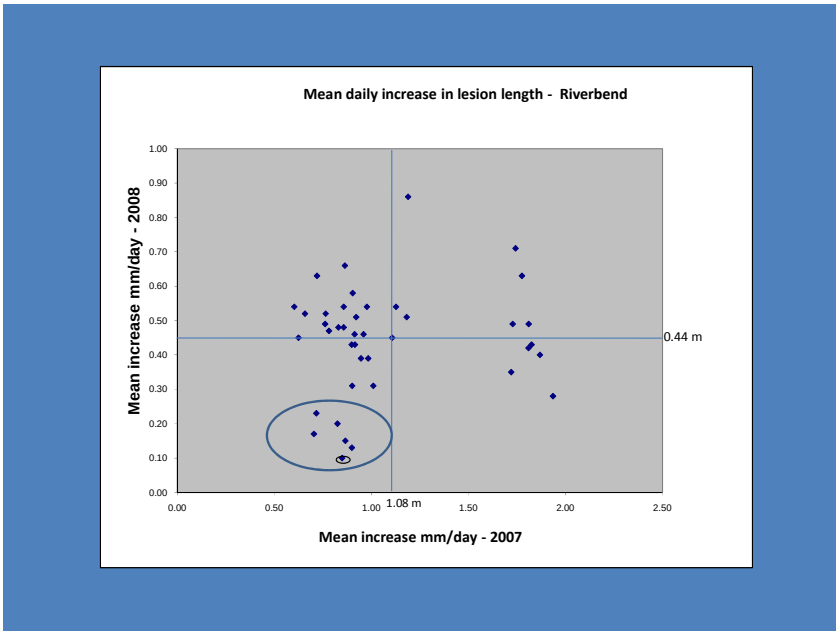
So far we have found that juvenile tissue roots more easily. Actively growing side shoots on suckers will root within four weeks. The method we are using has two steps. 1. In the first year graft candidate tree onto a potted rootstock. 2. In the second year, move the potted grafted plant into heated greenhouse in February. Take side shoots as cuttings in the first week of May and grow in greenhouse until the fall.



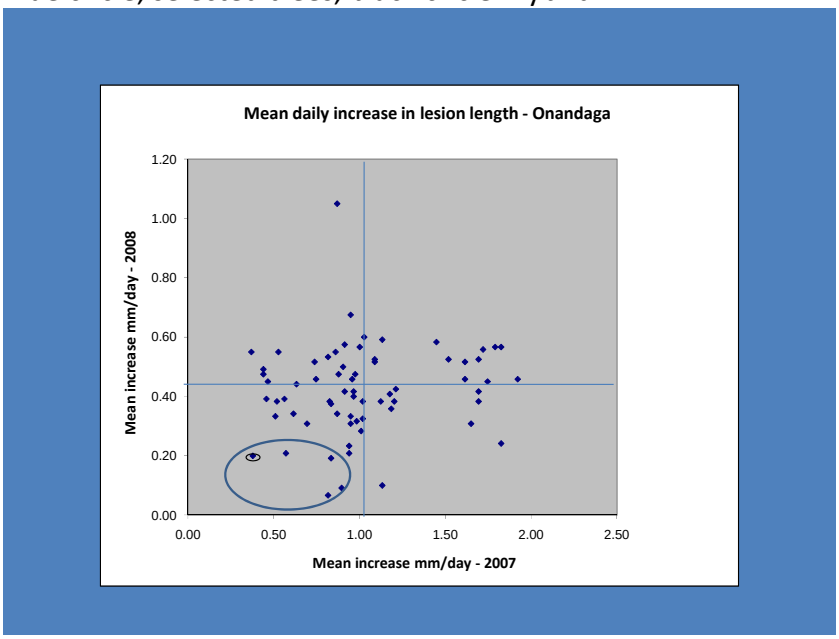
Left bar (yellow) – hybrid, right bar (white) – Canadian



Left bar (yellow) – hybrid, right bar (white) – Canadian



Blue circle, selected trees, black circle- hybrid



Blue circle, selected trees, black circle- hybrid

Sandra Anaganostakis, Connecticut Agricultural Experiment Station

Seed from CT crosses made in 2007 were raised in a Georgia nursery by S. Schlarbaum (UTn) and 1,650 seedlings were brought to CT in March 2009. Of these, 200 were given to a southern New York religious community for nut production and the rest were planted in CT. The former CT State Tree Nursery in Griswold, CT has been given to The Experiment Station, and a plot there was planted with 780 BC3 F2 and BC2 X BC3 seedlings as a seed orchard.

John Carlson, Pennsylvania State University

Genomic Tool Development for the Fagaceae *Project* (John E. Carlson, Abdelali Barakat, Scott DiLoreto, Alex Choi, Norzawani Yassin, and Tyler Wagner, The Schatz Center for Tree Molecular Genetics, School of Forest Resources at Penn State). This project is a collaboration with Ron Sederoff (PI), and Dahlia Nielsen and Chris Smith at NC State University, Bill Powell and Kathleen Baier of the College of

Environmental Science and Forestry at Syracuse, Paul Sisco and Fred Hebard at TACF, Sandra Anagnostakis at the CAES, Bert Abbot, Stephen Ficklin, and Meg Staton at Clemson University, and Tom Kubisiak of the US Forest Service Gulfport, MS. Our group at Penn State conducted the cDNA sequencing for establishment of the EST databases at Clemson. We have also assisted in analysis of the sequence data, and in validation of the expression of candidate genes through RT-PCR.

In summary, we created deep EST databases for the Fagaceae species American beech (*Fagus grandifolia*), white oak (*Quercus alba*), northern red oak (*Quercus rubra*), American chestnut (*Castanea dentata*), and Chinese chestnut (*Castanea mollissima*). We produced over 2 million sequence reads in total by 454 pyrosequencing and Sanger capillary sequencing of cDNA libraries from various tissues of the five Fagaceae species. This provided over 447 Mb of new expressed gene sequences, from which we assembled a total of 91,325 transcript contigs tagging a wide diversity of gene functions in the 5 species, including biotic and abiotic stress responses and disease resistance. All sequences are available in the NCBI short reads database. The transcript contigs and their BLAST annotations of are available at the project website (www.fagaceae.org) hosted by CUGI (www.genome.clemson.edu*)*. The Fagaceae project team is developing Chinese chestnut (*Castanea mollissima*) as a model for genomics in the Fagaceae. The 40,039 unigenes we obtained for Chinese chestnut provide a solid foundation for building this model, which include thousands of complete or near-complete coding sequences. From the unigenes, hundreds of potential SSR loci and thousands of SNPs were identified at CUGI and NC State that can be used to create new high density genetic maps for Chinese chestnut, American Chestnut and AxC chestnut hybrids. The co-localization of expressed gene sequences will also permit the genetic and physical maps for Chinese chestnut to be fully integrated with each other. This project is supported by NSF grant DBI-PGRP-TRPGR 0605135, Ron Sederoff PI. A manuscript describing the project and all of the tools available at the project web portal is in preparation. We published a paper in which we compared the transcriptomes of Chinese chestnut and American chestnut in cankers versus healthy stem tissues (Barakat et al. 2009. Comparison of transcriptome from cankers and healthy stems in American chestnut (*Castanea dentata*) and Chinese chestnut (*Castanea mollissima*). BMC Plant Biology, 9:51. 11 pages). In this paper we reported that the putative functions of the American chestnut and Chinese chestnut unigenes cover a diverse set of molecular functions and biological processes, including a large number of genes associated with resistance to stresses and response to biotic stimuli. *In silico* expression analyses showed that many of the stress response unigenes were expressed more in canker tissues than in healthy stem tissues in both American and Chinese chestnut. The results from the NSF project provide many candidate blight resistance genes, and an ideal platform for sequencing of the Chinese chestnut genome, which will soon be initiated under the new Forest Health Initiative (www.foresthealthinitiative.org).

Ali Barakat has extended the transcriptome part of the project to look for microRNAs as well. Our in silico analysis of the Fagaceae EST database involved the development of an analysis pipeline from which 114 conserved miRNAs and 9 new, non-served miRNA families have been identified in American chestnut. In Chinese chestnut we have identified 172 Unique, conserved miRNAs in 19 miRNA families to date. These include miRNA targets for several genes involved in resistance to biotic and abiotic stresses, including disease resistance.

Gary W. Micsky, Pennsylvania State University

Extension Activities at Penn State

Volunteer Recruitment, Development, and Utilization

- February 27, 2009 “American Chestnut Site Selection and Aftercare Workshop” Tionesta, PA, **20 participants**
- March 21, 2009 “American Chestnut Site Selection and Aftercare Workshop” Mercer, PA, **20 participants**

- May 21, 2009 “Ohio River Watershed Challenge.”Restoring Native Species” American chestnut station, **73 participants**
- June 3, 2009 “American Chestnut Restoration, Science and Volunteers Making a Difference”, Sharon PA, **52 participants**
- July 18, 2009 PA Forest Stewards Annual Meeting, **2 sessions, 25 participants**

Instruction and hands-on activities demonstrating efforts of PA-TACF and PSU in developing blight resistant American chestnut. Topics included: history of American chestnut and impact of *Cryphonectria parasitica,* TACF breeding program, grower/site selection, orchard maintenance, inoculation and selection of suitable genetics, and opportunities for volunteers. Program evaluations indicated:

Sessions were useful for improving your outreach to other landowners: 4.83/5 (very helpful)
Sessions were helpful at improving your understanding of your forestland: 4.75/5 (very helpful)

- *June, 2009 “Chestnut Chatter” an extension mailing list developed in 2008 was adapted to a Penn State listserv in order to accommodate the need to quickly notify **36 trained volunteers** of program activities such as: pollination schedules, harvest dates, and other labor intensive activities.*

Over 126 hours of service involving 12 volunteers to date (September 3, 2009) including 3 Mercer County volunteers who assisted in leaf collection at Graves Orchard on 08.10.09

***Identifying Potential Sites/Growers for Outplantings*.** Participants at February 27 and March 21, 2009 workshops were given an opportunity to take home 10 open pollinated seed in exchange for agreeing to provide baseline follow up information regarding their success or failure in growing chestnut seedlings on their site.

Over 400 open pollinated seed were distributed to 35 individuals with follow up surveys beginning in September 2009. These surveys will be used to determine grower dedication and site suitability for future outplantings. Baseline data will include: % seed surviving, height of seedlings, weed and pest controls, tree protection, and problems encountered as of September 2009.

Two new test orchards including open pollinated and F1 seedlings established near West Middlesex, PA (over 80 seed planted). Demonstration orchard established near Tionesta, PA (10 Chinese, 10 OP American, 10 F1)

Locating and Collection of Local Genetic Materials

- Controlled pollination to produce F1 seed on three American chestnut trees in Haun Orchard, Sandy Lake, PA
- Balloon pollination of Beagle tree, Mercer, PA
- One article submitted to Mercer County Woodland Owners Association newsletter on Chestnut Gall Wasp.

Laura Georgi, Rutgers University

In previous years, I had reported making a genomic (BAC) library of Chinese chestnut and taking first steps toward constructing physical maps of regions on Linkage Groups B and F containing genes for resistance to blight. These first steps included designing oligonucleotide (“overgo”) primers corresponding to RFLP markers CD175 (on LG B) and CD 145 (on LG F), identifying BAC clones in the library that hybridized to these probes, obtaining limited DNA sequence from the BACs, and designing PCR (CAPS) markers that can be used for genotyping in place of the original RFLP markers. The BACs have been fingerprinted (HICF), and we plan to incorporate these fingerprints in the whole-genome physical map from the NSF Fagaceae project. This will anchor two regions of the project’s map to particularly important regions of the genetic map. In addition, it should extend the local physical maps I have obtained for these regions and improve the chances of completing contigs spanning the regions of interest. The NSF project has developed new genetic markers and is starting to genotype new mapping populations and also hybridizing BAC library filters to place these genetic markers on the physical map.

With luck, some of these markers will map to the regions of interest and assist in delimiting the DNA containing the resistance loci

Contrary to what I reported in 2007, the CAPS marker developed for CD145 was successful and maps to the expected location in the genetic map of the A x C F2 population. There was a breakdown in communication, and once that misunderstanding was detected and rectified, the marker performed properly.

Unusual segregation of genetic markers in interspecific crosses of American and Chinese chestnut raised concerns that the organization the genomes in the two species differs in ways that could affect introgression of resistance from Chinese chestnut into American genetic backgrounds. Meiotic figures of hybrid chestnuts display clear evidence of a chromosomal translocation in one of the species compared with the other (Nurul Faridi, pers. comm.), and genetic evidence points to involvement of Linkage Groups B and E. Chromosome-specific probes are needed to determine which chromosomes are involved in these rearrangements. The BACs previously isolated are candidates for *in situ* hybridization probes for Linkage Groups B and F, and a 5S ribosomal DNA probe serves as a probe for Linkage Group E.

To obtain probes for identifying all twelve chromosomes, I screened my genomic library with additional genetic markers (Table 1). These markers included six more RFLPs (in addition to the two used previously), four isozymes plus the cystatin gene, and six microsatellites (SSRs) mapped in the A x C F2 population. This left me short of my goal of two probes per linkage group. In an effort to make up the deficiency, I used ESTs that mapped in European chestnut to linkage groups identified as probable homologs to the desired linkage groups in the A x C interspecific map. Since the whole point of this exercise is to elucidate genome rearrangements between species, obviously the genetic locations of these (European) markers in particular will have to be confirmed in the A x C map before they can be used confidently to identify the linkage groups to which I have tentatively assigned them.

Table 1. Molecular Probes to Identify Chestnut Chromosomes by *In Situ* Hybridization

LG	Locus	Type of marker	BACs	Number of HICF contigs
A	CD192	RFLP	14	1
A	PGI	Isozyme	17	1
B	CD175	RFLP	7	1
B	SKD	Isozyme	17	1
C	GM49	RFLP	13	1
C	PGM	Isozyme	12	1
D	CsCAT02	SSR	8	3
D	QpZAG58	SSR	18	1
E	5SrDNA	-	n.a.	n.a.
E	CCMC39433	EST[1]	11	1
F	CD145	RFLP	13	1
F	EMCs4	SSR	15	1
G	CD172	RFLP	13	2
G	CCMC04326	EST[1]	14	1
H	cystatin	CAPS	7	2
H	CD62	RFLP	18	4
I	CD143	RFLP	4	3
I	EMCs14	SSR	8	2
J	CD50	RFLP	10	2
J	GOT	Isozyme	11	1
K	CsCAT17	SSR	14	1
K	CCMC09685	EST[1]	13	1
L	EMCs15	SSR	20	1
L	CCMC40039	EST[1]	8	2

[1]EST markers have not been mapped in the A x C populations. They are from the European map, on linkage groups believed to be homologues of the ones listed here.

These BACs have been sent to Tom Kubisiak to prepare for use in chromosome Fluorescent In Situ Hybridization (FISH). They have also been subjected to HICF fingerprinting (so they can be incorporated into the Fagaceae project's physical map) and have been end-sequenced. In preparing the clones for distribution, I detected previously-overlooked cross-hybridizing BACs that were positive for probes CD143 (LG I) and CsCAT02 (LG D). Consequently, these BACs will probably not be suitable for their intended use as chromosome-specific probes for FISH.

Many of the BAC fingerprints assembled into single contigs corresponding to their hybridization history. The parameters used were quite stringent (tolerance = 3, final cutoff 1e -35), and the hybridization data were NOT used in assembling the contigs. The BAC end-sequences will be mined for SSRs and Single-Nucleotide Polymorphisms that can be used to confirm the genetic locations of the clones. A number of BAC ends contained sequences corresponding to the proper probes, for example, EMCs4.

Now that peach genome sequences are being assembled, the Chinese chestnut BAC end sequences can also be used in comparisons of genome organization in these two tree species. Depending on the extent of conservation, the peach genome sequence could be very useful in addressing problems in chestnut.

I have collected tissue from a small (51 seedling) back-cross population segregating for resistance to *Phytophthora cinnamomi* that Joe James and Steve Jeffers screened last summer. This material has been sent to Saucier MS for genotyping so the resistance locus can be mapped.

2009 Update. The BAC end sequences from this project provided an opportunity for additional exploration of the degree of conservation of synteny between chestnut and poplar. We had sets of BAC clones associated with eight pairs of linked markers in chestnut, at genetic distances ranging from 2 cM to 15 cM. Once again, sequences from BACs hybridizing to a given marker showed some tendency to

find matches that clustered in the poplar sequence. However, only the most closely-linked pair of genetic markers in our collection (CD145 – EMCs4) detected matches on the same linkage groups in poplar (VIII and X). Thus synteny with poplar may be conserved over map distances of 2 but not 6 to 15 cM in chestnut.

As it happens, CD145 and EMCs4 are very near a blight-resistance QTL, at about 3 and 1 cM, respectively. Chestnut sequences around the two loci match sequences separated by about 1.2 Mbp in the poplar sequence. How much farther does the conservation of synteny extend? If we go out on a limb a bit, and continue along the poplar sequence in the indicated direction, we encounter a number of genes that have annotations suggestive of a possible function in disease resistance: a dirigent-like sequence, a myb-109-like sequence, and a coi-1-like sequence. Some people think that dirigent-like proteins are involved in lignification, though there is some controversy about this; myb-109 appears to be a transcription factor; and coi stands for coronatine-insensitive, involved in jasmonic acid signaling. If homologues of any of these are found mapping next to EMCs4 in chestnut, they would be candidates for the resistance QTL; if any proves to be THE QTL, we'd have played a successful round of molecular golf.

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

This report presents progress made on four specific milestones that pertain to two of our three project objectives: 1) complete genetic map of Chinese and American chestnut; 2) test the translocation hypothesis in *Castanea*; 3) refine regions around blight resistance QTL; and 4) refine genetic linkage and genome sequence maps for *C. parasitica*

Milestone 1: complete genetic map of Chinese and American chestnut (2009). Considerable progress was made on this milestone over the past year. Approximately 350 simple sequence repeat (SSR) markers and 1536 single nucleotide polymorphisms (SNPs) were identified and used to construct genetic linkage maps for the parents of two different intraspecific Chinese chestnut crosses. The crosses used for genetic mapping were Vanuxem x Nanking (189 progeny) and Mahogany x Nanking (189 progeny). These populations were created by The American Chestnut Foundation (TACF) and the Connecticut Agricultural Experiment Station (CAES), respectively, as part of the National Science Foundation (NSF) Project: Genomic Tool Development for the Fagaceae. Highly promising candidate markers for genetic analysis were chosen from sequence data collected on cDNA libraries developed for both Chinese and American chestnut as part of the NSF Project. Criteria for choosing candidate markers included: observed polymorphism within specific cDNA libraries, total # of sequence reads suggesting the presence of polymorphism, allele quality scores, frequency of alleles, gene function, and marker design criteria. To date, ~250 SSRs and ~1100 SNPs were used to construct genetic linkage maps for Chinese chestnut. The maps consist of 12 linkage groups and represent each of the 12 known homologous chromosomes. The genetic length of the Chinese chestnut genome was estimated to be ~725-750 cM (recombination units) with an average spacing of ~0.5 cM between markers. Since all of the genetic markers were developed from expressed genes (ESTs), the maps graphically represent the genetic location of genes with potentially known function. Of the 1300+ genetic markers placed on the maps ~1100 have an assigned function based on BLAST values at exp<e^{-10}. Thus, a functionally annotated map of Chinese chestnut is now available for use in comparative studies with other species in

the family Fagaceae as well as with other more distantly related species such as Arabidopsis, Poplar, and Eucalyptus.

Our efforts are now focused on constructing a genetic linkage map for the American chestnut genome. Informative, i.e., polymorphic, SSRs and SNPs are currently being identified and will be used to construct genetic linkage maps for a single intraspecific American chestnut cross (182 progeny). The cross being used for mapping is GMBig x Horn that was created by TACF as part of the NSF project. Our goal is to place as many of the markers that were mapped in Chinese chestnut onto maps for American chestnut in an effort to maximize colinearity of the maps between species. Depending upon observed heterozygosity in the American chestnut parents we may have to identify and develop additional SNPs polymorphic in American chestnut cDNA libraries. Again, to maximize colinearity between species maps, our goal will be to target polymorphisms in the same genes mapped in Chinese chestnut. This milestone should be completed in late 2009 to early 2010.

Milestone 2: test the translocation hypothesis in *Castanea.* Once genetic linkage maps for both Chinese chestnut and American chestnut have been developed we intend to use the linkage data and marker colinearity (or lack-there-of) to test the hypothesis that the genomes of Chinese chestnut and American chestnut are structurally different due to translocations and or inversions which have occurred since their divergence from a common ancestor. The presence of major structural genomic rearrangements are important as they have implications for breeding blight resistant American chestnut using the backcrossing approach, especially if genes for blight resistance are located in or near these rearrangements. Previous genetic linkage data suggested that some structural differences may exist between these two genomes and some preliminary cytological data currently supports this hypothesis (refer to Figure 1 below). However, currently we do not know which chromosomes are involved in the apparent translocation/s. Genetic linkage data (and maps) for both species will be the definitive proof needed to confirm this hypothesis and determine the chromosomes involved. Figure 2 (below) shows an example of how genetic linkage data will be used to test this hypothesis. This milestone should be completed in early 2010.

Figure 1. Cytologically observed quadrivalent in interspecific F1 meiocytes

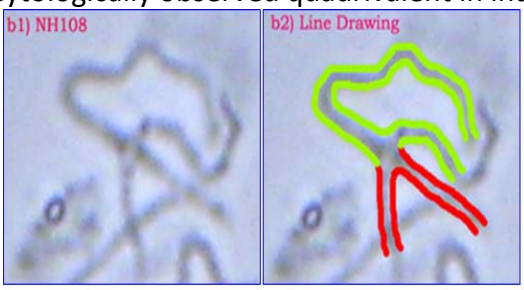
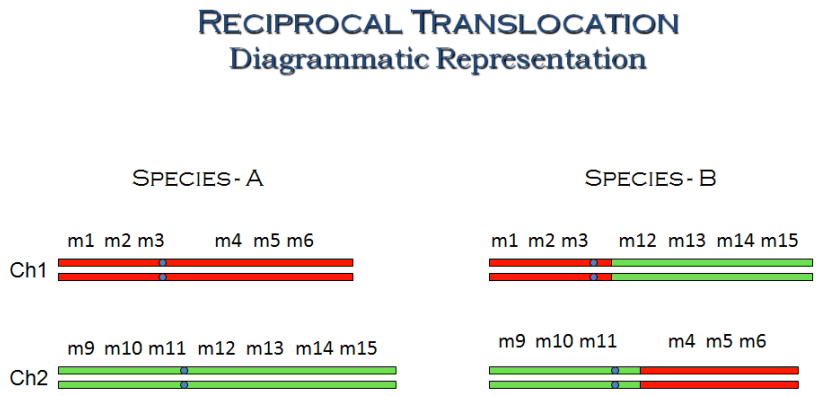


Figure 2. Diagramatic of translocation using genetic markers

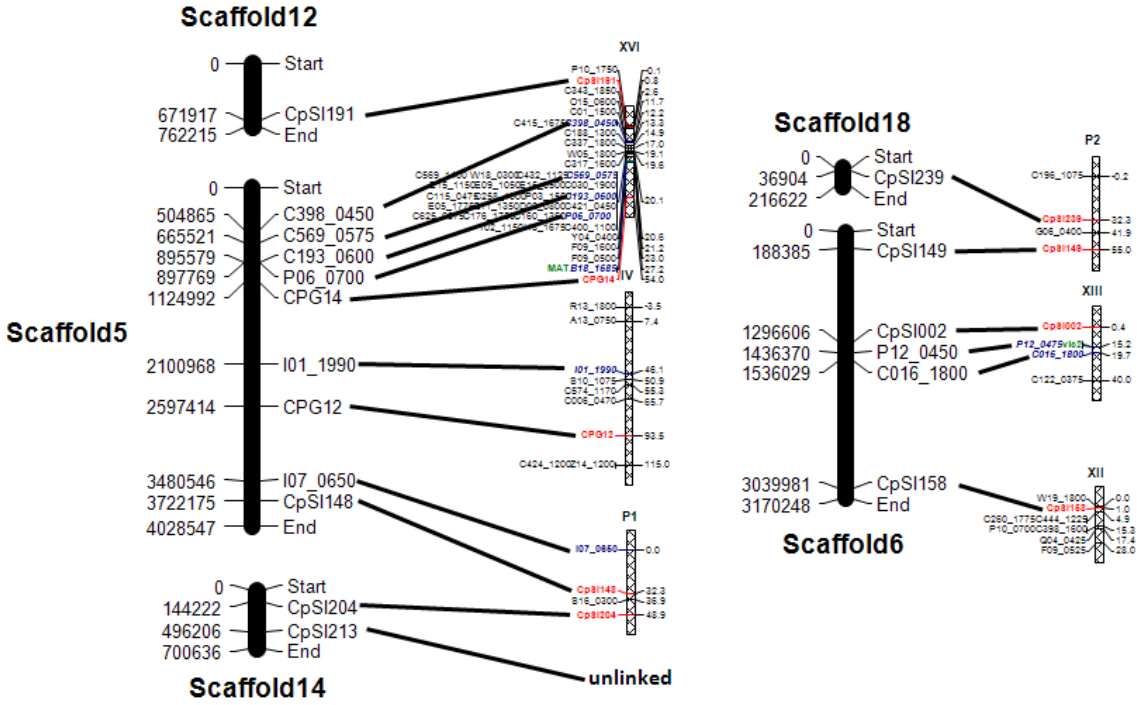


Milestone 3: refine regions around blight resistance QTL. The ultimate goal of the chestnut community is to identify the specific genes responsible for conferring resistance to the chestnut blight fungus, *Cryphonectria parasitica*. We have identified two approaches to laying the foundation for identifying causal genes: 1) fine map previously defined disease-resistance QTL in the F_2 mapping population created in the 1990s; 2) perform an association test between markers and phenotypes in large BC_3/BC_4 populations available to TACF. For Approach 1, stored DNA from ~85 – 90 individuals representing an F_2 between Chinese (Mahagony) and American chestnut parents are being used for mapping additional markers to those previously placed on a previous genetic map. We are currently screening the parents of the F_2 cross previously used to map blight resistance QTL. Our goal is to place these QTL within the context of our "new" genetic marker framework. Thus, only informative, i.e., polymorphic, markers will be genotyped against the progeny array of stored DNAs. Additional progeny from this cross have been created as part of the NSF project but have not been inoculated or phenotyped yet. Ultimately these additional progeny will be used to refine QTL map locations. This milestone should be completed by the end of 2010. As part of a new project, The Forest Health Initiative, the association mapping approach will be pursued and used to confirm the existence of currently known QTL as well as to identify potentially "new" QTL for resistance.

Milestone 4: Refine genetic linkage and genome sequence maps for *C. parasitica*.
A previous genetic linkage map for *C. parasitica* was developed and published in 2006. The genetic linkage map was incomplete in the sense that it existed of more than the expected nine linkage groups/chromosomes observed cytologically. Thus, a concerted effort has been made to mine the genome sequence of *C. parasitica* isolate Ep155 for additional genetic markers useful for genetic mapping. A complete genetic linkage map for *C. parasitica* will be useful for: 1) finishing the genome sequence of *C. parasitica* (which currently consists of greater than the nine expected chromosomes); and 2) for localizing and eventually identifying genes involved in particular traits of interest to the community such as vegetative incompatibility genes and genes influencing the formation of various pigments – although any segregating trait could potentially be localized. Over the course of the last 2 years, the entire genome sequence of *C. parasitica* has been mined for SSRs markers. Our first goal was to use newly identified markers near the ends of the 36 genome sequence scaffolds in an attempt to use linkage data to suggest which scaffolds might be on the same chromosome. Using this approach, linkage data suggested that 12 of the sequence scaffolds could be joined into six "new" scaffolds (refer to examples in Figure 3). This data has since been used by the Department of Energy's Joint Genome Institute and the version 2 genome build is now consistent with the genetic linkage data. Our second goal was to develop additional markers spaced evenly across the *C. parasitica* genome in an attempt to obtain a more complete genetic linkage map. Such a map would be invaluable for localizing genes influencing any traits (segregating phenotypes) of particular interest to the community. Primer pairs for an additional ~150 SSR markers evenly spaced across the genome have been developed and we are in the process of placing these markers on the current genetic linkage map. Our hope is that these

markers will reduce the number of linkage groups to the nine expected based on cytological observation. This milestone should be completed by the end of 2010. The sequencing of additional *C. parasitica* isolates, SG2-3 and Ep146, should yield data about informative SNPs and it is anticipated that these markers will provide complete coverage of the genome and a resolution far greater than that possible with SSRs.

Figure 3. Examples of joined genome sequence scaffolds using genetic linkage data



William A. Powell, SUNY-ESF

In 2006 we planted the first four transgenic American chestnut trees in the field and only one survived. In 2007, we planted 17 additional transgenic trees, all of the same event, Wirsig. All but one survived. In 2009, we planted 220 transgenic American chestnut trees, representing seven different events. An approximately equal number of trees are being maintained in the green house or growth chambers. The difference in production abilities is due to two new growth chambers and further refinement of the regeneration techniques. We can now produce transgenic American chestnut trees in less than 18 months with a high survival rate.

These improvements have allowed us to begin environmental impact studies on the transgenic American chestnut supported by the USDA's BRAG program. In this study, transgenic American chestnut trees are being compared to traditionally breed chestnut trees grown under open field and shelterwood settings. Impacts on mycorrhizae, insects, and plants in the planting sites are being monitored.

These improvements have also allowed us to collaborate with researchers on the NSF *Fagaceae* genomics project and the new Forest Heath Initiative to enhance the number and sources of genes to be tested. Therefore, in addition to the genes we previously developed into vectors, new genes and gene promoters from Chinese chestnut are becoming available. Due to the shear number of genes and the number of events needed to test each gene, new methods are being developed as an early pre-screen for blight resistance. We will be presenting preliminary results from one new type of prescreening protocol and also an overview of the current research. In addition, below are individual research summaries written by our lab personnel.

Kathleen Baier, SUNY-ESF

Differentially expressed genes in American and Chinese chestnut. American chestnut (*Castanea dentata*) was once a dominant tree species throughout the forests of the eastern United States. The species, which is susceptible to infection caused by *Cryphonectria parasitica,* was nearly destroyed when the exotic fungus was introduced into the United States over 100 years ago. Closely related Chinese chestnut (*C. mollissima*) exhibits a natural resistance to chestnut blight and the fungus causes only superficial cankers. The aim of this study was to examine species-specific genetic differences related to chestnut blight. To isolate genes unique to or more highly expressed in Chinese chestnut, an interspecific subtractive cDNA library was constructed by suppression subtractive hybridization (SSH) from Chinese and American chestnut canker margin tissue. Resulting Chinese chestnut library clones represented genes commonly induced during plant defense response to infection, including genes for beta-1,3-glucanase, phenylalanine ammonia-lyase and NADH respiratory burst oxidase. Of 126 sequenced library clones, 7.9% were identified as a gene for a laccase-type polyphenol oxidase (PPO). A gene for this laccase exists in the genomes of both chestnut species, yet northern blot hybridization and RT-PCR results show higher expression in stems of Chinese chestnut. An assay to detect PPO activity in stem tissue indicated that Chinese chestnut has significantly higher PPO activity. Plant laccases are suspected to play a role in phenolic metabolism and lignification and may therefore be important in plant defense response against pathogens. Genes highly expressed in Chinese chestnut would be good candidates for marker selection or genetic modification of American chestnut. Three plasmids with a gene for laccase, alone and in combination with other defense genes, have been constructed for transformation into American chestnut to determine if they can improve resistance to *C. parasitica.*

Andy Newhouse, SUNY-ESF

Co-transformation and insert copy number in transgenic American chestnut. Transforming American chestnut trees with resistance-enhancing genes is a promising strategy in the fight against chestnut blight. In the current study, American chestnut somatic embryos have been co-transformed with two separate Agrobacterium vectors. This allows a marker gene (i.e. pGFP) to be incorporated independently from the resistance-enhancing gene(s), and potentially bred out in future generations. Stably co-transformed embryos have been grown into shoots, multiplied in tissue culture, rooted, and acclimated. Resistance-enhancing genes currently being tested in whole plants include oxalate oxidase (OxO, from wheat) and a synthetic antimicrobial peptide called ESF39. The presence of these transgenes has been confirmed with PCR, but that doesn't reveal how many copies of the gene have been inserted into the host genome. The best way to determine this is with a Southern blot, in which chestnut genomic DNA is bound to a nylon membrane, and hybridized with a radiolabeled probe for the gene of interest. The probe binds wherever there is a complimentary sequence, so the number of bands from a given sample represents the transgene copy number. Of the five Southern blot-tested transgenic lines containing OxO, three have a single copy of that gene, one has two copies, and one has three copies. Multiple copies of a gene may increase expression levels, but can also lead to confounding effects known as "gene silencing". Further, single copy transgenic events are preferable if the organism will be bred at some point, as the inheritance patterns will be much simpler and more predictable. The fact that most of these events are single copy is encouraging, as many transformations result in much higher copy numbers. Expression patterns of each transgenic line have been tested, and field trials are currently underway to observe ecological and environmental interactions of transgenic chestnuts.

Lilibeth Northern, SUNY-ESF

Testing oxalate oxidase expression and regeneration of transgenic American chestnut. Restoration of the American chestnut via resistance to the blight is our team goal. Recently we have been using a technique called “co-transformation” with two simultaneous plasmids; one plasmid has

GFP (a reporter gene) plus a second plasmid that contains the gene of interest (OxO, which enhances resistance against the chestnut blight). This technique has given us promising results. We have gotten a number of transgenic shoots containing both plasmids, and they grow and multiply nicely.

As with any transformation system, it is necessary to check the expression of the gene of interest at several steps before we get the plants rooted and ready to plant in the field. To monitor the expression of the OxO gene, we use an oxalate oxidase assay. We test the transformed embryos, as soon as they grow shoots, as well as later, when they are big enough for being planted in pots. This assay is fast (it gives results in a couple of days), easy to perform, and it helps us to confirm that we have a transgenic chestnut with the putative resistance-enhancing gene being expressed as expected. The test gives us a visual expression coming from the sample (5mm leaf-disc). According to the presence of the staining color (black), its location and abundance, we classified the plant as having vascular induced expression, wounded induced expression, or non-expression at all.

I have been involved in all of these steps, from growing untransformed embryos to planting the transgenic chestnuts in the field. One of my main contributions to the chestnut team is to validate that the transgenic chestnuts (with the OxO gene) are all checked with the oxalate oxidase assay mentioned above. At the moment, we are also aiming to a more accurate test, on which the OxO staining expression can be related with a quantifiable protein measurement. Hopefully soon we will have some results to share in this area.

This year I have also been involved with the regeneration of new transgenic plants that contain both, the OxO gene and the ESF39 antimicrobial peptide gene. This “pyramid” gene construct is predicted to give higher and more durable resistance. There are already about a hundred plants rooted and growing nicely in both, the greenhouse and growth chamber. Many more still in process of in vitro multiplication and getting them ready for the field.

Amelio Bo Zhang, SUNY-ESF

Comparing constitutive OxO expression to regulated OxO expression in Transgenic American chestnut. During the past year, I have constructed a plasmid vector called p35S-OxO that has a 35S promoter driving the OxO gene. This promoter is a constitutive promoter, which means we should be able to observe OxO activity throughout the transgenic American chestnut plant. This will give us a better understanding of how this gene works in the tree, and will also act as a control to compare with the VspB promoter in the pTACF3 vector. The VspB promoter only expresses in vascular tissue, and would be a good candidate to put in those transgenic American chestnut lines being prepared to go through the deregulation process.

With these vectors made, together with pGFP, I have done many transformations to put these genes into American chestnut. So far, I have produced 10 confirmed events with pTACF3 and pGFP, 7 confirmed events with pGFP only, 7 confirmed events with pWVK147 (an empty control vector) and pGFP. In addition, I have, more than 40 putative events with p35S_OxO and pGFP to be confirmed. When these events have been regenerated into whole plants, we will have many more transgenic American chestnut lines to be tested both at tissue culture stage and out in the field. In fact, there are some events in shooting and multiplication stage in tissue culture right now including 3 events with pTACF3, 5 events with pGFP only, and 2 events with pWVK147.

Katie D’Amico, SUNY-ESF

Ectomycorrhizae colonization of transgenic American chestnut roots. One approach to enhance blight resistance in American chestnut is to incorporate genes linked to disease resistance into the chestnut genome. These genes vary in their function and products, but act to combat the fungus either directly or indirectly. At SUNY-ESF, multiple transgenic lines have been developed using a number of genes thought to have the potential to enhance resistance to blight if expressed at appropriate levels. One of these transgenic lines contains the gene for oxalate oxidase, an enzyme that can be produced by

chestnut to enhance resistance to blight. While it is highly unlikely, there is a possibility that production of this enzyme could have unintended effects on ectomycorrhizae, the fungi that colonize the roots of the chestnut tree. Before any transgenic tree can be released into its native and natural environment, impact assessments must be performed to ensure that there are not any negative impacts on non-target organisms. In order to assess this potential impact on ectomycorrhizae, we are performing a greenhouse study using a variety of trees including the transgenic line of American chestnut containing the oxalate oxidase gene, non-transgenic American chestnut, Chinese chestnut, an American-Chinese hybrid, American beech and red oak. Using field soil as an inoculum, these plants will be grown in pots in the greenhouse. We will analyze root samples from a subset of these trees and compare levels of colonization by different fungal species using RFLP analysis. If the transgenic American chestnut falls in an acceptable range of fungal diversity comparable to the non-transgenic and the other *Fagaceae* trees, then it would seem that this specific transgenic line of American chestnut is not having a negative impact on non-target ectomycorrhizae. Using these types of studies, we will be able to easily gauge the potential impact of all of the transgenic lines of American chestnut on ectomycorrhizae.

Steve Jakobi, Alfred State College

Chinese and American x Chinese Chestnut hybrid planting (in association with Cheryld Emmons, Alfred University). This experiment compares the growth characteristics of year-old saplings of one American chestnut genotype, two Chinese chestnut genotypes and two intercrosses produced from F1s backcrossed to American chestnut grown under different planting distances. Seeds were planted in May 2008 on the campus of Alfred State College in Alfred, NY in a randomized block design. Replicate blocks (4 for each spacing) consisted of five seeds from each genotype planted either one, two or three feet apart in rows that were spaced nine feet apart. Not all of the seeds either grew or survived the winter; therefore growth parameters were measured on four to six trees per spacing distance per genotype from July to October 2009. Effects of genotype, spacing and genotype by spacing interactions were determined using the univariate GLM procedure of SPSS.

Due to a great deal of natural variation, no significant effects of genotype, spacing or genotype-by-spacing interactions were found for photosynthetic rate, dark respiration rate, number of stems, height of tallest stem, or total stem length. Chinese chestnut genotype 1 had a significantly higher survival rate (70%) than all other genotypes (37-45%). The difference in survival is most probably due to the depth of planting, as re-planting in May 2009 resulted in nearly equal germination. Survival was not affected by spacing or genotype-by-spacing interactions. As with morphology, the growth parameters measured in this experiment do not differ significantly between American chestnut and American x Chinese chestnut hybrid intercrosses.

Neal VanAlfen, UC Davis

The quarantine time period for the imported chestnut trees at FPMS (Foundation Plant Materials Service) was over last winter. They planted the potted trees in the field. Three trees were lost in the process but all varieties are still represented.

The Varieties include 'Marrone Comballe', Marrone di Chusa Pesio', Marrone di Marradi', 'De Coppi', 'Qing', 'Luvall's Monster', 'Eaton' and 'Campbell NC.'

<u>OBJECTIVE 2.</u> To evaluate biological approaches for controlling chestnut blight from the ecological to the molecular level by utilizing knowledge of the fungal and hypovirus genomes to investigate the mechanisms that regulate virulence and hypovirulence in *C. parasitica*

Dennis Fulbright, Michigan State University

Vegetative compatibility structure for three Michigan populations was determined winter 2008 through spring 2009. Sampled populations included two initially healthy populations (Missaukee

healthy and Leelanau) with recent disease colonization and one epidemic population, Stivers, where disease has been common for over 20 years. The number of VC groups found at each population were as follows: Leelanau, 1; Missaukee healthy, 2; and Stivers, 3. Samples from Leelanau and Missaukee encompassed 50 trees of 1—10cm diameter at breast height (DBH). Fifty canker samples were obtained from Stivers but only from the southern half of the population to leave an internal control for the hypovirulence inoculation study and to look at the spread of the virus through time in that population.

Each VC background was infected via pairings of hypovirus GH2 with a virulent culture and was used to re-inoculate the same chestnuts in the same populations in spring 2009. A total of 35 cankers were treated at Leelanau, 49 at Missaukee healthy (5 VC2, 34 VC1 and 10 were a mixture of groups), and 100 at Stivers (59 VC1, 25 VC2, and 16 VC3). Cankers will be re-inoculated in spring 2010 if needed. Growth and survivorship of these inoculated trees will be compared to controls to test if GH2 is an effective virus to use as a bio-control for chestnut blight fungus infections. Other work continues on determining VC structure at 4 additional Michigan populations.

Donald Nuss, University of Maryland Biotechnology Institute, Center for Biosystems Research
DOE/JGI Community Sequencing Program: Project Proposal

Project Title: Genome sequencing of the chestnut blight fungus *Cryphonectria parasitica* (submitted March, 2006, approved June, 2006).

Proposer's Name:

Donald L. Nuss, Center for Biosystems Research, University of Maryland Biotechnology Institute

Other Proposers:

Alice C. L. Churchill, Department of Plant Pathology, Cornell University
Michael G. Milgroom, Department of Plant Pathology, Cornell University

Other participants:

John Carlson, Pennsylvania State University, USA
Baoshan Chen, Guangxi University, PR China
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Dae-Hyuk Kim, Chonbuk National University, South Korea
Thomas Kubisiak, USDA Forest Service, USA
Myron Smith, Carleton University, Canada
Neal Van Alfen, University of California, Davis, USA
Michael Wingfield, University of Pretoria, South Africa

Abstract:

Cryphonectria parasitica, the chestnut blight fungus, is responsible for epidemics that caused the destruction of tens of millions of mature chestnut trees in forests of North America and Europe during the first part of the 20th century. The discovery of a group of RNA viruses, now classified in the family Hypoviridae (hypoviruses), that reduce the virulence (hypovirulence) of this pathogen stimulated intensive research into the potential of using fungal viruses for the biological control of fungal disease. Subsequent epidemiologic and population genetic studies have established the chestnut/*C. parasitica*/hypovirus pathosystem as the textbook example of both the consequences of accidental introduction of an exotic organism and of hypovirulence-mediated biological control of fungal pathogens.

Interest in *C. parasitica*, hypoviruses and their interactions now extends well beyond disease control potential. The development of a robust *C. parasitica* transformation protocol and of hypovirus reverse genetics led to the establishment of a biologically relevant experimental system with the rare

capacity for efficiently manipulating the genomes of both a eukaryotic virus and its host. Scientists studying this system have also made significant contributions to the current understanding of mycovirus-host interactions, fungal population genetics, mechanisms underlying fungal pathogenesis and fungal signal transduction pathways. Very recent advances with this system are providing important new insights into the role of RNA silencing as an antiviral defense mechanism in fungi and the impact of viruses on fungal vegetative incompatibility systems and secondary metabolism. Thus, availability of the *C. parasitica* genome sequence, the first for an Ascomycete tree pathogen, would greatly accelerate the efforts of an active and growing research community that address a broad range of important fundamental and applied research topics.

The objectives of the proposal are to: A) assemble an 8-10 X sequence coverage for the genome of *C. parasitica* strain EP155; B) perform an automated and directed annotation of the assembled genome sequence; and C) provide a web-accessible database of the *C. parasitica* genome sequence with necessary tools for mining and comparative genome studies by the research community.

Scope of Work:

Genomic DNA will be prepared from the most widely used and best characterized *C. parasitica* strain, EP155. Expectations from JGI include the generation of approximately 400Mb of raw sequencing reads required for 8 – 10 X coverage of the estimated 40-Mb *C. parasitica* genome, sequence assembly and initial automated annotations. We also request assistance in organizing a gene annotation jamboree with members of the *C. parasitica* research community. The annotation process will be aided by the availability of two large *C. parasitica* EST libraries that have been generated by laboratories in the U. S. and China and a linkage map; annotation will be aided further by comparisons with the completed genomes of the phylogenetically related fungi *Neurospora crassa* and *Magnaporthe grisea*.

Update on JGI Genome Sequencing Project: The JGI released version V2 of the *C. parasitica* genome assembly on July 10, 2009. The 39 scaffolds in the original version were reduced to 26 scaffolds by joining some of the original scaffolds based on linkage map information provided by Tom Kubisiak and on additional sequencing efforts. The table below shows the correspondence between new and old scaffolds. Five of the new scaffolds contain two teleomers and are the size predicted for complete chromosomes. Scaffold 8 likely corresponds to an intact Chromosome 9. So, the assembly is progressing nicely.

The new version is undergoing re-annotation. This means that the manual annotation has been frozen. The portal is still accessible, but it is not possible to enter any annotation data. The protein IDs will be retained in the new version only for those genes that have been annotated or are in the process of being annotated. Un-annotated genes will receive a new ID number. Igor indicated that the new annotated version would be available by end of September-early October.

Cryphonectria Build V2

Comparison of Build Version1 scaffolds to build Version2 scaffolds

Version 2 Scaffold	> 1MB Size MB	Teleomers	Version 1 Scaffold(s)
1	7.4	2	1
2	5.5	2	12+5+14
3	5.2	2	3+17
4	5.1	2	2
5	4.3	1	4
6	3.9	1	8+10

7	3.3	1	6+18
8	3.2	2	9+15
9	2.9	1	7
10	1.3	1	13+16
11	1	1	11

Chromosome 9

Our petition to JGI for 454/Solexa/Illumina re-sequencing the *C. parasitica* strain SG2-3 genome was accepted. Strain SG2-3 was chosen for re-sequencing because a) it has been paired with the reference sequence strain EP155 (high virulence strain) for extensive inoculation of progeny of the TACF Chestnut resistance breeding program as a low virulence strain, b) it is of the opposite mating type from EP155, and c) a collection of ascospore progeny are available from a cross with the reference sequence strain EP155 and the progeny are being tested for virulence levels on chestnut trees. Resequencing of the SG2-3 genome will allow the generation of a single nucleotide polymorphism (SNP) map that will provide the means to build a very dense linkage map with the EP155 X SG2-3 progeny.

The SG2-3 re-sequencing is predicted to:

1) Aid in finishing the *C. parasitica* genome.

2) Provide SNP map that will allow identification of pathogenicity genes and vegetative incompatibility genes and provide the opportunity to connect genome-sequencing effort with the Chestnut tree blight-resistance breeding program.

3) Provide new insights into Ascomycete mating locus organization and function.

Angus Dawe, New Mexico State University

Post-translational modification of phosducin like protein BDM-1. Phosducin-like proteins are conserved regulatory components of G-protein signaling pathways, which mediate a variety of physiological processes. Widely expressed throughout the eukaryotic genomes, they have been identified as positive regulators of Gβγ complex assembly in mammals. Genetic studies revealed that *C. parasitica*, a plant pathogen and causative agent of chestnut blight disease, contains three G-alpha, one G-beta, one G-gamma subunits as well as phosducin-like protein BDM-1 that have important roles in pigmentation, sporulation and virulence. Deletion of either Gβ subunit or BDM-1 produces identical phenotypes, including reduced accumulation of the Gα subunit. Additionally, the Gβ subunit is not detectable in absence of BDM-1. Evidence from mammalian systems suggested that the regulatory role of BDM-1 may be controlled by protein kinase II (CK2) mediated phosphorylation. We confirmed that BDM-1 can be phosphorylated by CK2 and identified five putative CK2 phosphorylation sites. Consequently, substitution of serine residues at the serine sites with either alanine or aspartic acid has revealed that CK2-mediated phosphorylation at only two of these sites is physiologically relevant. Interestingly, co-expression of the BDM-1 mutants in the presence of myc-tagged Gβ resulted in changes in accumulation of overexpressed Gβ subunit indicating that phosphorylation BDM-1 by CK2 influences G-beta stability.

cis-regulator regions controlling expression of bdm-1 and cpgb-1. As reported in 2007 and 2008, we have designed a reporter system based on GFP to examine genomic regions required for the control of expression of these two genes. We now have 7 reporter constructs and validated expression controlled by regions of 1461, 1232, 989, 808, 455, 277 and 141 and bp upstream of *bdm-1*. Additionally, we have 5 further constructs that use genomic pieces of 800, 555, 374, 216 and 165 bp upstream of *cpgb-1*. All but the smallest fragments in each case are capable of driving significant GFP expression.

Hilary Boyer has also examined the effects of infection with hypovirus on EGFP expression. Amanda Kemp (who left in 2008) observed that hypovirus infection greatly affects the presence of the reporter, but only when driven by larger upstream fragments from 5' to *bdm-1*. Smaller fragments (<

500 bp) are still capable of driving reporter transcription, but are not affected by virus infection. Examining the sequence showed that this area was C-rich (40 – 45 %) and therefore led us to investigate the potential for methylation changes. By employing bisulfite sequencing techniques (treatment with sodium bisulfite causes non-methylated cytosines to be converted to thymine, methylated residues are protected) Hilary has been able to reproducibly document extensive changes to DNA methylation as a result of hypovirus infection: 232 of 349 cytosine residues in more than 800 bp immediately 5' to *bdm-1* appear methylated in the uninfected strain, only three residues were methylated in the presence of hypovirus This has highlighted the potential for epigenetic influences on genome structure mediated by the hypovirus.

Major impacts on the primary metabolism of the plant pathogen Cryphonectria parasitica by the virulence-attenuating virus CHV1-EP713. See publication (attached): A. L. Dawe, W. A. Van Voorhies, T. A. Lau, A. V. Ulanov and Z. Li. (2009). Microbiology, in press (available online as doi: 10.1099/mic.0.029033-0)

Controlled gene expression in the plant pathogen Cryphonectria parasitica by use of a copper-responsive element. See publication (attached): K. L. Willyerd, A. M. Kemp and A. L. Dawe (2009). Appl. Environ. Microbiol, 75: 5417-5420.

William MacDonald, West Virginia University

Introduction of hypoviruses at West Salem, Wisconsin W.L. MacDonald and M.L. Double (in cooperation with J. Cummings-Carlson, Wisconsin Department of Natural Resources; D.F. Fulbright and A.M. Jarosz, Michigan State University; and, M. Milgroom, Cornell University)

The stand of American chestnut in West Salem became infected with chestnut blight in the late 1980s after 100 years of blight-free growth. Hypovirus introduction (individual canker treatment) was conducted from 1992-1997 (700 cankers on 133 trees received inoculum). From 1998-2002 hypovirus introduction was halted. In 2001, due to a large increase in the number of cankers in the stand, twelve permanent plots were established in three regions of the stand representing differing levels of disease (Disease Center; Front and Beyond the Front). Hypoviruses were reintroduced in 2003; annual treatment has continued through 2009. Two methods were employed to evaluate whether biological control had been initiated: (1) punch treatment of individual cankers as done initially; and (2) wounding of trees by inoculating scratch wounds to the bark surface to create reservoirs of inoculum. One-half of the plots in each region were punch treated and the remaining half were scratch-treated. Approximately 25% of the trees in each plot were untreated to assess tree-to-tree spread.

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

Trees that received hypovirus treatment in 1996-1997 but were not incorporated into the plot system of 2001 were located and sampled in June 2009 by Sally Dahir (Wisconsin DNR) and Jim Savarino. Approximately 50 living trees that were last treated with hypoviruses 12-13 years ago were located (based on GPS data). Many had no visible cankers; some were dead. Bark samples (12 per canker) from 50 cankers were taken from old wounds on 29 historically treated trees (if cankers were visible) or from cankers on their nontreated sprouts. Thirty-nine of fifty cankers (78%) yielded hypovirulent isolates and fourteen of those cankers (38%) yielded 10, 11 or 12 plugs of CHV1 hypovirus (of 12 bark samples per canker). These historically treated trees not only have harbored CHV1 hypovirus for more than a decade, but the CHV1 hypovirus has proliferated in many cankers.

Data tables from the above cankers are listed below. Morphology of cankers (based on a subjective 1-4 rating system [1=callus and no stroma; 2=callus and some stroma; 3=callus and moderate stroma; 4=no callus and heavy stroma]) was correlated to the amount of hypovirus detected in a canker.

Of the 50 cankers sampled, 74% of the cankers were rated as #1 or #2 (callus with little or no visible stroma).

Number of Cankers by Canker Rating			
#1	#2	#3	#4
24	13	8	5

Average Isolate Morphology (of 12 bark plugs)			
	Virulent	Euro 7	Non *C. parasitica*
#1 Rated Cankers	1.5	7.1	3.3
#2 Rated Cankers	0.8	6.3	4.8
#3 Rated Cankers	7.3	2.3	2.5
#4 Rated Cankers	10.2	0	1.8

Transmission of hypoviruses into conidia from cankers at West Salem, WI S.A. Naymick (Summer Undergraduate Researcher), W.L. MacDonald and M.L. Double. Cankers on thirty-two trees were sampled from areas of the West Salem chestnut stand representing differing histories of hypovirus treatment in order to assess transmission of hypovirus into conidia. Overall, approximately 2,800 individual conidia were assayed from 96 pycnidia. In the area with the longest history of hypovirus treatment (Disease Center), 43% of individual pycnidia contained hypovirulent conidia. The production of hypovirus-infected conidia from these pycnidia ranged from 13%-66% with an average of 37%. This was in sharp contrast to an area of the stand with a shorter history of hypovirus treatment (Front), in which only one of twenty-four cankers harbored pycnidia that contained hypovirulent conidia. No cankers in the Beyond the Front area contained hypovirulent conidia.

The biological control potential of Cryphonectria parasitica strains containing an infectious cDNA copy of the hypovirus CHV1-Euro7 W.R. Rittenour, M.L Double, W.L. MacDonald (in cooperation with D.L. Nuss-University of Maryland Biotechnology Institute). This study, initiated in 2004, was designed to evaluate whether transgenic *C. parasitica* strains containing a cDNA transgene encoding the viral genome of CHV1-Euro7 show greater potential to biologically control chestnut blight than their cytoplasmically-infected counterparts. Three treatments were employed to compare transgenic hypovirulent (TG), cytoplasmic hypovirulent (CH), and virulent (V) strains. To produce ascospore inoculum, naturally occurring and artificially established punch-initiated cankers were spermatized by painting cankers (PI) three times each summer (2004-2009) with a conidial mixture containing MAT-1 and MAT-2 mating types of the appropriate treatment strain (TG, CH, or V). Conidial inoculum was produced by inoculating scratch wounds (SI) made to the bark with a mycelial slurry of the appropriate treatment strain (TG, CH, or V). Non-treated trap (T) trees were left to monitor natural canker development as well as hypovirus spread. Tree condition and natural canker establishment were assessed for all trees in August of each year. As of August 2009, there were 172 natural cankers in TG plots, 101 in CH plots, and 100 in V plots. Cankers were sampled, when detected and each November, to determine the hypovirus infection status of the thallus. Although the purpose of the spermatization treatment was to produce ascospores, many treated cankers also acquired hypovirus from the treatment inoculum and have begun to produce callus.

Some significant findings this past year include the increased detection of hypoviruses, both in the canker thallus and perithecial outcrosses. Beginning in 2007 and continuing through 2009, hypoviruses have been detected in cankers on trap trees, both in the thallus and ascospores. Transgenic inoculum has been detected in not only TG plots but also in CH and V plots. Ascospore colonies were assessed by pigmentation and morphology. Over seventeen-thousand individual ascospore colonies were examined from 800 individual perithecia in 2008. To date, 73,856 individual ascospores from 2,617

perithecia have been examined. Hypovirulent ascospore isolates were collected from 79% of the spermatized cankers in TG plots. From those cankers, 52% of the perithecia yielded transgenic ascospores. Outcrossing to inoculum (2006-2008), on a canker and perithecial basis, is shown in the following table.

	2006		2007		2008	
Trt.	% Cankers outrcrossed to treatment inocula	% Perithecia outcrossed to treatment inocula	% Cankers outrcrossed to treatment inocula	% Perithecia outcrossed to treatment inocula	% Cankers outrcrossed to treatment inocula	% Perithecia outcrossed to treatment inocula
CH-PI	40%	18%	54%	28%	41%	23%
CH-SI	33%	12%	21%	11%	44%	25%
CH-Trap	0%	0%	13%	3%	5%	1%
TG-PI	76%	47%	79%	52%	66%	33%
TG-SI	59%	25%	36%	15%	53%	28%
TG-Trap	11%	9%	17%	8%	5%	1%
V-PI	50%	15%	52%	21%	45%	15%
V-SI	62%	34%	54%	27%	72%	44%
V-Trap	30%	13%	9%	2%	0%	0%

Tree mortality, as of July 2009, was greatest in the virulent plots (87%), followed by TG plots (69%) and CH plots (58%).

Transmission of hypoviruses into conidia from cankers in the transgenic research plots in WV M.A. Malone (Summer Undergraduate Researcher), W.L. MacDonald and M.L. Double. Asexual inoculum production (conidia) from 39 cankers was examined; 14 from transgenic, 6 from virulent and 19 from cytoplasmic plots. Overall, approximately 1,700 conidia were examined from 98 individual pycnidia. Hypovirulent conidia were recovered from 44% of pycnidia in TG plots and 20% of pycnidia in the CH plots. From hypovirulent pycnidia, hypoviruses were transmitted to 46% and 25% of conidia in the TG and CH plots, respectively; an average of 36% when data from both treatment plots are combined. In addition to examining hypovirus transmission, bark samples were taken from each canker to ascertain the status of the underlying fungal thallus. Hypovirulent conidia were detected in thirty-seven percent of cankers that had an underlying hypovirulent bark thallus. Of the cankers which had a virulent thallus, only one produced hypovirulent conidia. Of the 119 hypovirulent conidia, all but one originated from stroma with an underlying bark thallus that was hypovirulent.

Michael Milgroom, Cornell University

Graduate student: Marin Brewer; Collaborators: Kiril Sotirovski (University Ss. Kiril i Metodij, Republic of Macedonia), Cyril Dutech and Cécile Robin (INRA, Bordeaux)

Note: Milgroom currently has no research funds or active projects on *Cryphonectria parasitica*, chestnut blight or hypoviruses.

Continuing projects (but no significant progress since last year):

Worldwide population structure of *C. parasitica*: Cyril Dutech and Cécile Robin (see 2008 report for details).

Heterokaryons in *C. parasitica* in Europe: Kiril Sotirovski. As reported last year, we found a few isolates in Italy and Macedonia that had two alleles at multiple marker loci. Analysis of hyphal tip and single-conidial isolates have shown conclusively that these are heterokaryons. In 2009, we published a paper reporting these results (Milgroom M.G., Sotirovski K., Risteski M., Brewer M.T. 2009. *Fungal Genet. Biol.* 46: 849-854).

Alice C.L. Churchill, Cornell University

Collaborator: Clay C.C. Wang (University of Southern California, Los Angeles, CA)

Note: Churchill currently has no research funds for research on *Cryphonectria parasitica* or chestnut blight or hypoviruses.

Characterization of a secondary metabolite pathway in *Cryphonectria parasitica*. As reported last year, a natural product biosynthetic pathway encoded by a polyketide synthase gene, *PKS1*, was disrupted using *Agrobacterium tumefaciens*-mediated transformation methods. HPLC and mass spectrometry (MS) analyses of extracts of control and *PKS1* gene knock-out strains have provided a preliminary identification of the polyketide natural product produced by the *PKS1* pathway. Confirmatory analyses are in progress. Basic manual annotation of all PKS and nonribosomal peptide synthetase genes in the *C. parasitica* genome has been completed at the DOE-JGI-CSP *C. parasitica* genome web portal.

Neal VanAlfen, UC Davis

CHV1 was shown to co-localize in infected cells primarily with fungal trans-Golgi network vesicles containing the Kex2 protease. We report here the cloning, analysis and possible role of the *C. parasitica* Kex2 gene.

C. parasitica Kex2 gene sequence analysis showed high similarity to other ascomycete kexin-like proteins. Southern blot analyses of CpKex2 showed a single copy of this gene in the fungal genome. In order to monitor the expression and evaluate the function of CpKex2, antibodies were raised against expressed protein and Kex2 silenced mutants were generated. Western blots indicate that the Kex2 protein was constitutively expressed. Growth rate of the fungus was not significantly affected in Kex2 silenced-strains, but these strains showed reduced virulence, reduced sexual and asexual sporulation, and reductions in mating and fertility. The reduced virulence was correlated with reduced Kex2 enzymatic activity and reduced relative mRNA transcript levels as measured by real time RT-PCR. These results suggest that secreted proteins processed by Kex2 are important in fungal development and virulence.

Previous work reported that CHV1 elements co-fractionate with *trans*-Golgi network membranes of *C. parasitica.* We have also previously reported that the cell surface hydrophobin cryparin accumulates in the same subcellular fractions as the CHV1 elements, and when gradients are loaded with equal dry weight equivalents, the viral containing strain shows a much higher concentration of cryparin in these same fractions. This vesicle fraction accumulates approximately 2.5 to 5 fold greater concentration in the CHV1 infected strain. Using the GFP reporter gene inserted into the coding region of cryparin, fluorometric quantification confirmed the vesicle accumulation data. Microscopy studies of the GFP constructs revealed expression of the fusion protein in discreet intracellular bodies approximately 100 nm in diameter. In contrast, the same construct containing CHV1 showed a very different pattern of accumulation. Fluorescence was seen most prominently near or in the plasma membrane and septal regions. Co-localization with FM4-64 and Calcafluor confirmed plasma membrane and septal localization of the fusion protein in the viral containing strain.

<u>OBJECTIVE 3.</u> To investigate chestnut reestablishment in orchard and forest settings with special consideration of the current and historical knowledge of the species and its interaction with other pests and pathogens.

Sandra Anaganostakis, Connecticut Agricultural Experiment Station

Two new diseases have become a problem at Lockwood Farm in Hamden. An anthracnose caused by a *Pseudomonas* was widespread this year, and was often associated with shot holes caused by insect damage. A suspension of the bacterium from pure culture caused the disease when sprayed on seedling American chestnuts in a growth chamber. The second was a canker disease resulting in heavily calloused lumps and was found on three trees. Surface sterilized tissue always yielded a *Pseudomonas* when sampled. The symptoms resemble photographs in a paper from Japan (Takanashi and Shimizu, 1989, Ann. Phytopath Soc. Japan <u>55</u>:398-403) reporting *Pseudomonas syringae* pv. *castanea* on Japanese chestnut trees.

Seedlings of *Castanea ozarkensis* from eastern Oklahoma were collected and grown by S. Schlarbaum (UTn) and planted in Hamden, CT in 2004. Dormant stems of three of these *C. ozarkensis*, one *C. mollissima*, and one *C. dentata* were cut in February 2009 and inoculated with *C. parasitica* strains Ep 155 and Weakly, and the stems incubated at room temperature in plastic boxes. The pathogens had formed lesions after 10 days on *C. dentata*, but had grown little on the *C. mollissima* or the *C. ozarkensis*. Strain Weakly appeared to be more virulent than Ep155 on stems of one of the *C. ozarkensis* trees. Tests of stems from more of the trees will be conducted in the winter of 2009-2010.

Lynne-Rieske Kenney, University of Kentucky

The Asian chestnut gall wasp, *Dryocosmus kuriphilus*, is a potentially devastating pest of chestnut that causes round or globular twig, shoot, and leaf galls on actively growing shoots. Galls act as vegetative sinks that influence the nutrient and defense status of developing tissues. Galling reduces tree vigor, prevents normal shoot development and can cause tree mortality. Galling also prevents infested shoots from producing new shoot growth and reproductive flowers, thereby reducing or eliminating nut production. Chestnut production and chestnut restoration efforts throughout the eastern USA are threatened by the persistent spread of this exotic, invasive insect. The Asian chestnut gall wasp was first observed in the USA in 1974 infesting Chinese chestnut near Bryon, GA, and has since spread north to Pennsylvania and Maryland, and westward to central Kentucky. My research program has been evaluating the mechanisms associated with gall formation and characterizing associates of the Asian chestnut gall wasp in eastern North America, to more fully understand gall development and what factors may regulate gall wasp populations.

Selected plant signaling compounds are being evaluated to assess their role in gall formation and defense. Ethylene, abscisic acid, and jasmonic acid, and associated inhibitors, have been evaluated. Exogenous ethylene applications reduced gallmaker fitness and source strength, and compromised defenses in both American chestnut and F1 hybrids. Exogenous abscisic acid applications moderately affected gallmaker fitness, source strength, and defense in F1 hybrids only. Jasmonic acid increased gall fitness and defense in Chinese chestnut, whereas the JA inhibitor (DEICA) decreased gall fitness and defense on American chestnut. The natural enemy complex and parasitoid recruitment has been characterized, with seven parasitoid species documented as being associated with the gall wasp. Interactions between the most prevalent native parasitoid, *Ormyrus labotus*, and an introduced parasitoid, *Torymus sinensis*, which was introduced for Asian chestnut gall wasp control, are being evaluated. We are also evaluating the extent to which galling alters the susceptibility of infested chestnut to subsequent herbivory. Galls may alter concentrations of nutrients and defensive compounds in leaves and other plant parts, and so can affect subsequent preference and performance of herbivores utilizing the same food source. Our ultimate goal is to gain an understanding of the ecological interactions, dispersal patterns, and mechanisms regulating gall wasp populations in eastern North America.

Dennis Fulbright, Michigan State University

At this juncture, we now have the ability to differentiate five cultivars commonly grown in Michigan based on genotype rather than tree morphology. To increase the informative value of SSR markers for the identification of chestnut species and cultivars growing in Michigan, additional microsatellite markers are being evaluated. Currently, four of the five SSR loci evaluated resulted in unique SSR profiles for the cultivars tested, ‘Colossal’, ‘Nevada’, ‘Benton Harbor’, ‘Eaton’, and ‘Okei’ (Table 1). The CsCAT1 primer profiles are unique for ‘Nevada’, ‘Benton Harbor’, and ‘Eaton’. However, the CsCAT1 profiles of ‘Colossal’ and ‘Okei’ are the same, however, these cultivars can be differentiated when EMCs15 and EMCs32 are used as primers. The CsCAT2 and EMCs15 profiles were inconsistent for ‘Benton Harbor’ and ‘Eaton’, respectively. The highest number of inconsistent profiles was observed for the CsCAT16 locus. However, CsCAT16 was useful for genotyping ‘Colossal’ and ‘Benton Harbor’.

EMCs32 was the least useful marker for the cultivars tested since it resulted in monomorphic genotypes for 'Colossal', 'Nevada', 'Benton Harbor' and 'Eaton', but it plays an important role differentiating 'Colossal' from 'Okei'.

In the summer of 2007, four different cultivars ('Nevada', 'Okei', 'Eaton, and 'Benton Harbor') were crossed with the cultivar 'Colossal' in isolated plots and the resulting F1 progeny genotyped via SSR loci. At least 50 seedlings were generated per cross. The leaves were collected and stored at -80ûC. DNA was extracted and purified for each seedling sample and stored at -80ûC as described above. We determined the efficiency of each SSR marker to differentiate cultivars by analyzing the probability of identity (PI), and the power of discrimination (PD) that were calculated after processing the SSR data with *Identity 4.0* software.

As shown above, four primers of the five SSR loci evaluated produced amplicons of the expected size range and resulted in polymorphic bands for all four F1 populations and the parents (Table 2). Although CsCAT2, CsCAT16, and EMCs15 were polymorphic, these loci were non-informative for 1 to 3 of the F1 populations included in this study.

Of the 5 primers tested, only CsCAT1 produced clear polymorphic bands for the four F1 progenies and their parent plants included in this study (Table 3). The number of alleles for the CsCAT1 locus was 6. The probability of finding two identical genotypes (PI) using CsCAT1 was similar to the reported value for *C. sativa* cultivars (0.1304 and 0.160). The power with which this locus excludes an erroneously assigned individual for being the parent of an offspring was moderate, with a value of Q = 0.4698. However, the power of discrimination (PD), the probability that two random genotypes could be distinguished by their CsCAT1 profile was high, with a value of PD = 0.72. Together, the PI and Q values suggest, as expected, that a single SSR locus, while useful, is not sufficient for parentage analysis.

Adam Dale, University of Guelph, Canada

American chestnut is endangered in Ontario. The American chestnut is considered an endangered species in Canada and is covered nationally by the Canadian Species at Risk Act. This Act essentially designates its status and provides for a recovery plan. The recovery plan can be found at http://www.sararegistry.gc.ca/document/default_e.cfm?documentID=648. In Ontario, the Endangered Species Act was enacted in 2007 and came into force last year. http://www.mnr.gov.on.ca/en/Business/Species/2ColumnSubPage/STEL01_131232.html. Essentially, 'the Bill prohibits killing, harming, harassing, capturing, taking, possessing, collecting, buying, selling, leasing, trading or offering to buy, sell, lease or trade a member of the species.' We are negotiating with the Ontario Government on how the Act affects what we are doing.

Paul Sisco, The American Chestnut Foundation, Asheville

Abstract. In March, 2009, Fred Hebard suggested that data from five years of experiments indicated the presence of a single, partially-dominant factor in Chinese chestnut that confers resistance to *Phytophthora cinnamomi* Rands. Scientists working with the National Science Foundation's Fagaceae project are now trying to fine-map this locus with hopes of eventually identifying and cloning the gene or genes involved in resistance.

The principal scientific work going on with chestnut in North and South Carolina is screening for resistance to *Phytophthora cinnamomi*, the causal organism of Ink Disease. Both Chinese and Japanese *Castanea* species have resistance to this disease, whereas the North American and European species do not.

In 2004, Joe James, M.D. of Seneca, SC, a volunteer with The Carolinas Chapter of The American Chestnut Foundation, recruited the assistance of Steve Jeffers Ph.D., a Phytophthora expert at nearby Clemson University to set up screening experiments to identify seedlings that were resistant to *P. cinnamomi* among families originating from TACF's Meadowview Research Farms. In these experiments, seedlings are grown up at close spacing in sterile soil in large tubs and then inoculated with zoospores of

the disease organism. At the end of the growing season, all seedlings are dug up and rated on a 0 – 3 scale, with "0" showing no disease symptoms and "3" being dead.

Fig. 1. Joe James in green smock goes over planting plan with Inga McLaughlin, post-doc, while Steve Jeffers and post-doc Jae-soon Hwang plant another tub in the background. Seedlots are divided into replicates for the seven tubs, and colored stakes mark off the dividing line between families to be screened.

Fig. 2. Root systems of (left) Chinese and (right) American chestnut seedlings after exposure to *Phytophthora cinnamomi* for a summer season.

Most seedling families screened in these experiments originated with either the 'Clapper' or 'Graves' sources of blight resistance. 'Clapper' and 'Graves' were BC_1 trees developed by the chestnut breeding programs of the USDA and Conn. Agric. Expt. Stn., respectively, and were used as starting points for TACF's backcrossing program. If there is a single factor for resistance coming from Chinese chestnut, then there was a 50/50 chance that 'Graves' and 'Clapper' would inherit the gene from their F_1 parent. Data so far indicate that 'Graves' did get the factor whereas 'Clapper' did not. Thus resistance to *P. cinnamomi* is segregating in seedlings derived from 'Graves' but not in seedlings derived from 'Clapper'.

In 2007, Paul Sisco initiated a cross between a 'Nanking'-derived F_1 tree at Meadowview, VA (GL158) and a long-surviving American chestnut tree in Kentucky (the Adair County tree). Michael French of the Kentucky Chapter – TACF made the cross, and about 60 seed were produced in a difficult drought year. Of these, about 50 germinated in the James/Jeffers tub test in 2008 and were screened for resistance to *P. cinnamomi.* Before inoculum was added to the tub, leaf material from all seedlings was collected by Laura Georgi of Clemson and quickly stored in a -80^{o} freezer at the university. After exposure to the root rot organism, about half the seedlings died, consistent with the hypothesis of a single locus for resistance. Tom Kubisiak of the Southern Institute of Forest Genetics is now extracting DNA from the leaf material collected by Dr. Georgi as well as from the GL158 and Adair County parents of the family. If

there is a single factor for resistance segregating in this small family, Dr. Kubisiak should be able to identify the linkage group on which the factor resides. Meanwhile, larger BC_2F_2 and BC_3 families were created at Meadowview this summer for screening in 2010. These may allow for fine-mapping of the resistance locus, using the numerous genetic markers created by the NSF Fagaceae Genomics Project.

Respectfully submitted,
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October 2009