NE-1333 Technical Committee Meeting
Biological Improvement of Chestnut through Technologies that Address Management of the Species, its Pathogens and Pests

Nittany Lion Inn, State College, PA
September 7-9, 2018

Attendance:

Kentucky:	Albert Abbott (University of Kentucky)
Maryland:	Bruce Levine (University of Maryland)
Massachusetts:	Sandra Anagnostakis (Connecticut Ag. Exp. Station)
Michigan:	Andrew Jarosz (Michigan State University)
Mississippi:	Angus Dawe, (Mississippi State University)
New Jersey:	Bradley Hillman, administrative advisor (Rutgers University)
New York:	Linda McGuigan, Hannah Pilkey (SUNY-ESF), Steve Jakobi (Alfred State)
North Carolina:	Paul Sisco (TACF®, Asheville), Jason Payne (Woodland Restoration, Bear Creek)
Pennsylvania:	John Carlson (chair), Sara Fitzsimmons, Kim Steiner, Nicole Zembower, Tatyana Zhebentyayeva, Steven Hoy, Maureen Mailander (Pennsylvania State University), Ivor Knight, Michael Campbell, Emily Dobry, Kara Dobson (Penn State Erie, The Behrend College), Gary Micsky (PA TACF)
South Carolina:	Steve Jeffers, Andrew Gitto (Clemson University)
Tennessee:	Hill Craddock, Kirsten Hein, Taylor Perkins, Trent Deason, Erin Taylor, William Scott Smith (University of Tennessee, Chattanooga)
Vermont:	Kendra Collins (TACF®, Burlington), Yurij Bihun (Shelterwood Systems)
Virginia:	Fred Hebard, Laura Barth (TACF®, Meadowview), Laurel Rodgers, Teresa Zielinski, Bailey Hamilton (Shenandoah University)
West Virginia:	Matt Kasson (chair-elect), Donald Nuss, William MacDonald, Mark Double, Amy Metheny (West Virginia University)

The meeting was called to order by Chairman John Carlson at 8:15 am on 7 Sept 2018 at the Nittany Lion Inn, State College, PA. Carlson indicated that Mike Messina, head and professor of the Department of Ecosytem Science and Management, would join the group at a later time.

Table of Contents, 2018 NE-1333 Meeting, Nittany Lion Inn, State College, PA

Speaker	Subject	Page
Anagnostakis, Sandra	Valuable chestnut germplasm in Connecticut	3
Ray, Charles	The Penn State Xylarium	6
Pilkey, Hanah	Transgenic American chestnut outcrossing: 2018 outcross updates and long-term pollen storage research	7
McGuigan, Linda	An update on the Transgenic American Chestnut Program at SUNY-ESF	7
Double, Mark	B3F3 planting	8
Carlson, John	Chestnut genome sequencing project activities	9
Carlson, John	Breeding and field trials	10
Carlson, John	Wood phenotyping and extension	12
Carlson, John	Outreach	12
Tchatchoua, Dorothy	Breeding strategy of genetic stocks of *Castanea sativa* in Greece	13
Zhebentyayeva, Tetyana	Genetic mapping the resistance/susceptibility response in chestnut	16
Double, Mark	NE-140 pathogenicity study from 1985	16
Double, Mark	Backcross orchard for assessment of host resistance combined with hypovirulence	17
Rodgers, Laurel	Comparison of the fungal microbiome in chestnut trees resistant to *C. parasitica* to those that are not resistant to *C. parasitica*	18
Hamilton, Bailey and Zielinski, Teresa	Comparison of fungi species growing inside American, Chinese and hybrid chestnut trees	21
Kasson, Matt	Two-year study of hypovirus transmission by engineered super donor strains into a vegetative incompatibly diverse natural population of the chestnut blight fungus *Cryphonectria parasitica*	22
Metheny, Amy	Treatment delivery of superdonor fungus	23
Jarosz, Andrew	Michigan chestnut census	24
Jarosz, Andrew	Intra-canker variability for secondary fungi	24
Dawe, Angus	Identifying targets of the VIB-1 transcription factor, a component of the vic-mediated signaling pathway	27
Hebard, Fred	Why hypoviruses have resulted in remission of blight in Europe but not in North America	32
Sisco, Paul	Combining genomic with phenotypic selection in TACF's backcross breeding program and methods for incorporating transgenes to enhance blight resistance	33
Jarosz, Andrew	Commercial chestnut orchards	36
Jeffers, Steve	Phytophthora root rot on American chestnut	37
Jeffers, Steve	Screening of Hybrid American Chestnut Seedlings for Resistance to *Phytophthora cinnamomi*	37
Gitto, Andrew	Can excised twigs be used to identify resistance to *Phytophthora cinnamomi* in hybrid chestnut trees?	40
Deason, Trent	Conservation and collection of *Castanea dentata* in the south	41
Perkins, Taylor	Genome-wide SNP data and morphology uncover cryptic diversity in the chinquapins	43
Nelson, C. Dana	Overview of US Forest Service research	48
Dobry, Emily	Isolation of *Gnomoniopsis smithogilvyi* from trees of *Castanea* exhibiting atypical cankers.	49
Business Meeting		50
Publications 2017-2018		52

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

Sandra Anagnostakis, Connecticut Agriculture Experiment Station (Emeritus)

Valuable chestnut germplasm in Connecticut. Anagnostakis reported that she retired two years ago and moved to Waltham, MA. She travels to CAES one day to week.

The renewed interest in chestnut tree breeding has raised questions about the availability of valuable germplasm. There have been chestnut trees of several species in this country for a long time (*C. sativa*, 1773; *C. crenata*, 1876; *C. mollissima*, 1903; *C. Henryi*, 1908; *C. seguinii*, 1918). The USDA Plant Introduction Office imported and numbered (PI #) many chestnuts, as did the Forest Pathology branch (FP #). The Connecticut Agricultural Experiment Station and a few private land owners have many very fine trees. In the 1930's and 40's, Arthur Graves and Donald Jones planted many of the trees now used in the U.S. for breeding and molecular biology experiments. Additions of species and hybrids were made by Hans Nienstaedt and Richard Jaynes, and I have added others over the last 50 years. Some of my favorites are listed here to illustrate the richness of the resource. Trees are in Experiment Station plantings (which include over 1000 trees) unless otherwise noted.

Chestnut count , CAES orchards, 2018		**Total**
Castanea dentata (Marshall) Borkhausen	American chestnut	250
Castanea crenata von Siebold & Zuccarini	Japanese chestnut	15
Castanea mollissima Blume	Chinese chestnut	100
Castanea sativa Miller	European chestnut	38
Castanea alnifolia Nuttall	Florida chinquapin	2
(*Castanea floridana*?)	Florida chinquapin	4
Castanea pumila Miller	Allegheny chinquapin	4
Castanea ozarkensis Ashe	Ozark chinquapin	57
Castanea henryi Rehder & Wilson	Chinese chinquapin	6
Castanea seguinii Miller	Chinese dwarf chestnut	2
Hybrids		878

The hybrids include those from the (1960's) thesis project of R. A. Jaynes, which are reciprocal crosses of all the species in our collection.

Older hybrids of special interest are:

- (*C. crenata* x *C. sativa*) x *C. dentata* FP #551-two trees planted 1931, one the "Smith hybrid" and one called "Hammond-86", both mother trees growing on Long Island, NY
- (*C. mollissima* PI #70315 x *C. dentata* FP #551) x *C. dentata* from Clinton Corners, NY, planted 1955, called "Graves"

Ortets

We have the ortets of the following cultivars:

- 'Sleeping Giant' *Castanea mollissima* PI #70315 x [(C. *crenata* X C. *sativa*) Smith hybrid x C. *dentata* FP #551], planted 1938
- 'Mahogany' *Castanea mollissima* PI #70315, planted 1930

- ‘Essate Jap’ [(S8 of Van Fleet, *C. pumila* x *C. crenata*) open pollinated seedling] x *C. crenata* PI #78626, planted 1935
- ‘Toumey’ *C. mollissima* PI #78672 x [(C. *crenata* x C. *sativa*) Smith hybrid x C. *dentata* FP #551], planted 1938
- ‘Lockwood’ [(*C. crenata* x *C*. *sativa*) Hammond hybrid x *C*. *dentata* FP #551] open pollinated (probably by *C. crenata*), planted 1947
- ‘Little Giant’ [(*C. mollissima* PI #70315 x C. *seguinii* PI #70317) x (*C. mollissima* PI #70315 x *C. seguinii* PI #70317)] open pollinated, planted 1971, a stable dwarf tree which produces dwarf seedlings
- ‘Hope’ sibling of ‘Little Giant’, planted 1971
- ‘King Arthur’ sibling of ‘Little Giant’, planted 1971
- ‘Sadie Hunter’ probably a seedling of ‘Sleeping Giant’, planted about 1970
- ‘Madison’ sibling of ‘Little Giant’, planted 1971

and grafted trees of the following cultivars:

- ‘Clapper’ (*C. mollissima* x *C. dentata*) x *C. dentata,* Russell B. Clapper
- 'Colossal' *C. crenata* x *C. sativa*, C.E. Parsons, from Fowler’s Nursery
- 'Eaton' seedling of ‘Sleeping Giant’?, A. H. Graves & R. A. Jaynes, CT
- 'Orrin' *C. mollissima* PI #108552, Peter Liu (Hang Chow, China), Orin S. Good’s orchard, PA

Species trees

Various important species trees on CAES land in Hamden, Connecticut unless otherwise noted:

Japanese chestnuts *Castanea crenata*

- Three trees planted in 1876, probably `Parsons' Japan' all on private land (First Congregational Church, Cheshire; Bee and Thistle Inn, Old Lyme; P.T. Barnum house, Bridgeport)
- PI #78626, seed from wild trees in Oguriyama, Amori-ken, Japan, planted 1933 (orchard tree)
- PI #104015, Nobeoka Eirinsho, Yokugomura, Higashi, Usuki-gun, Miyasaki-ken, Japan (32 deg. latitude, planted 1935 (orchard tree)
- PI #104016, Numakunai Eirinsho, Ippoimura, Iwate-gun, Iwate-ken, Japan, planted 1935 (timber tree)
- PI #113679, Iwate-ken, Japan, planted 1939 at the CT Arboretum in New London (orchard trees)

Seguine chestnuts *Castanea seguinii*

- one surviving tree of PI #70317, "Mo-lut-tsz" Chiuhwashaan, Anhwei, China, planted 1929 (dwarf species) and one cross (1998)

Chinese Timber Chinquapins *Castanea henryi*

- one tree of PI #104058, "Chu-Lee" or "Chun Lee" "pearl chestnut," Hsiaohsing, Anhwei, China, planted 1935

- five trees of GA 30 and two of GA 31 from seed produced at Callaway Gardens in Georgia, collected by R. W. Camp, planted 2011

Chinese chestnuts *Castanea mollissima*

- two trees of PI #70315, "hardy trees native to north-eastern China" planted 1929, one is Graves' 'Mahogany' (timber trees)
- PI #78744, "Tiger Paw" from the Fa Hua Ssu Temple near Beijing, planted 1932 (orchard tree)
- two trees of PI #104061, `Lui An' Chekiang Province, China (28-32 deg. latitude) planted 1935 (timber trees)
- four trees of PI #104063, `Kuei Lee' "large chestnut," Hsinteng, Chekiang Province, China, planted 1935 (timber trees)
- two trees of PI #39721, from Tientsin, China, planted 1916 at the Bartlett Arboretum in Stamford (timber trees)
- one tree of Frank Meyer's import PI #36666, from the Pingchuan region N.E. of Beijing, planted 1917 at the Bartlett Arboretum in Stamford CT (this orchard tree is the cultivar `Bartlett')
- grove of trees of PI #58602 from north eastern China, planted 1926 in Dayville. There are vigorously naturalizing.seedlings in surrounding fields(1992).
- 60 trees of 'Mahogany' x 'Nanking' (WL R1T15 PI #70315 and pollen from Greg Miller, PI #108552). These are from the Fagaceae Genetics Project and were planted in 2010.

European chestnuts *Castanea sativa*

- one tree from wild seed collected in the Black Forest in Germany, planted 1985
- 15 trees from four areas with wild chestnut near Bursa, Turkey, planted 1991
- 21 trees from six eastern areas along the Black Sea in Turkey planted 2008
- one tree from seed collected by Fred Paillet in the Cavcas Biosphere Reserve in the Caucasus Mountains of southern Russia, planted 1994

American chestnuts *Castanea dentata*

- about 250 trees, seedlings from Michigan, Wisconsin, New York, and Connecticut, kept alive by hypovirulence in the blight fungus population
- one graft of the tree "Scientist's Cliffs" from land of Flippo Gravett in Port Republic, Maryland; had measurable resistance to chestnut blight (see Anagnostakis, 1992) (Sandy doesn't think it is a *dentata*)
- 8 trees on Painter Hill Road, near Painter Ridge Rd., in Roxbury, CT, used in Experiment Station crosses almost yearly from 1948 to 1961, may have some resistance to blight

American Chinquapins

- *C. pumila*, one tree from Empire Chestnut Co. planted 2000, three trees from Germantown, MD planted 2014
- *C. ozarkensis*, 57 trees from the Ozark Plateau in OK, planted 2004 and 2005
- *C. alnifolia*, two trees from Lafayette County, FL planted 1995

- *C. floridana*?, four trees from New Port Richiey, FL planted 2011

These trees have been used by Experiment Station Staff and by The American Chestnut Foundation scientist Fred Hebard and others for crosses and experiments for many years. They will probably be here for future use as well. A list of the inventory of Experiment Station plantings (with maps) is available from the author upon request.

Charles Ray, The Pennsylvania State University

The Penn State Xylarium. Ray worked for in the wood industry for years and when he camp to Penn State he found the Xylarium (a herbarium that specializes in wood specimens) that was hidden and untouched since 1968. In 2013, Ray gave a presentation in Florida and he found and international wood collectors society. Upon returning from Florida, Ray wrote about his experience in a blog. The blog raised a lot of interest. The collection in 2013 contained about 4,000 specimens. The PSU Xylarium now contains about 15,000. This is half of the specimens of the SUNY-ESF collection that contains roughly 30,000 specimens. The USDA Forest Products Lab in Madison, WI has the largest collection in the US with about 100,000 specimens. The Penn State collection was begun in 1909 with 48 specimens.

Gary Carver, a member of the Maryland chapter of TACF, and their former chapter president, raised questions about the quality of wood in TACF's backcross trees. Thus, Ray is going to pursue Carver's question by looking at wood specimens from American, Chinese and backcross trees.

Xylaria are often used in historical preservation. Wood in Saint Patrick's cathedral is being replaced and Ray was asked to consult. A sliver of wood was given to Ray and it was determined that most of the framing in the cathedral was sugar pine from the Pacific northwest.

An individual on France has developed a technique of sanding and fixing wood that makes for excellent views under a stereomicroscope. Ray showed several examples of wood that was fixed in this manner. Red oak and American chestnut were compared. Northern red oak has very wide rays. American chestnut also has rays but they are only 1-cell thick. American chestnut has 2/3 the weight of oak. Thus, it is lighter but stronger.

Research uses of Xylaria:

- Genetic variation
- Chemical characterization
- Medical research
- Endangered species identification
- Woody plant systematics

Ray also is trying to conduct chemical characterizations. It was found that a compound from Pacific yew is an effective agent combatting breast cancer. Thus chemical characterization is useful in medical research.

Chestnut studies with Xylaria

- Validating chestnut in historical structures (i.e. Monticello)
- Examining historical specimens for genetic clues
- Comparison among wood specimens

To date, Ray has 30 specimens from *Castanea* and *Castinopsis*.

Hannah Pilkey, SUNY-ESF

Transgenic American chestnut outcrossing: 2018 outcross updates and long-term pollen storage research. Outcrossing the transgenic lead event, Darling 58 (D58), with wild-type mother trees in our experimental plots to produce transgenic offspring is one of our team's primary goals at this stage in the project. Through outcrossing, we increase genetic diversity and reduce founder effects. Outcrossing also provides an exciting opportunity to rescue remaining genotypes and rare alleles.

Thus far, we have outcrossed D58 with two mother trees to produce the first outcross generation (OC1). Transgenic pollen produced from the OC1 trees was used this field season to pollinate 16 mother trees, 12 of which are new. In total, we pollinated 736 female flowers. Given the hemizygous position of the OxO gene on the chromosome, approximately 50% of all pollinated nuts will inherit the gene for blight-tolerance. Unfortunately, a periodical cicada emergence occurred and many of the branches holding our controlled pollinated bags were lost to cicada damage. Despite the cicadas, we are still anticipating a great yield during harvest and expect transgenic offspring from each mother tree.

Pending government approval, SUNY-ESF plans on distributing transgenic pollen to those who are interested in performing their own outcrosses. In the meantime, we have been optimizing each step in our protocol for collecting and storing pollen. The objective of the pollen research is to maximize pollen viability while minimizing pollen grain loss to improve outcross efficacy.

In 2017, a long-term storage experiment was conducted. Two different storage containers (vials and slide boxes) were tested as well as three different desiccation times (4, 24, and 48 hours). We also compared two different freezer temperatures (-20°C and -80°C). After 8 months of freezer storage, the pollen viability from each sample was analyzed by assessing pollen tube germination. It was found that the most optimum storage protocol for American chestnut pollen is as follows: Collect pollen by stripping from catkin into a 20 ml glass scintillation vial with screw-top lid, use a canister with Drierite rocks to desiccate between 4-24 hours at 4°C, and move into a -80°C freezer.

This year, we also tested a pollen vacuum as a new collection method, originally developed by *Arabidopsis* researchers (Johnson-Brousseau & McCormick, 2004). The cordless and lightweight vacuum is attached to a nozzle with two separate filters. The outermost filter traps debris, while letting pollen pass through. The second filter traps pollen grains in a cluster. If the mesh filter can be properly desiccated and stored, it may serve as a convenient way to collect and distribute transgenic pollen samples in the future.

Linda McGuigan, SUNY-ESF

An update on the Transgenic American Chestnut Program at SUNY-ESF. *Small Stem Inoculations.* In the summer of 2018, three teams performed small stem inoculations at field sites in Syracuse, NY. Bill Powell inoculated first generation outcrossed (OC1) transgenic American chestnut seedlings. These trees contain the oxalate oxidase gene (OxO) driven by a 35S constitutive promoter. They were pollinated in the summer of 2016, planted in the spring

of 2017, and include the original D58+16001 and D58+16020 seedlings. Andy Newhouse, Dakota Matthews, Hannah Pilkey, and Josh Mott inoculated small tissue cultured derived trees that were in pots. These clonal trees came from the D58+16001 and D58+16020 lines. Finally, Erik Carlson inoculated tissue culture-derived trees containing the OxO gene driven by a wound inducible promoter (Win 3.12). For Bill's OC1 seedling inoculations, canker area was significantly different after 30 days, but no wilt was seen on the susceptible controls (n=3 Darling 58 OC1's, n=1 Darling 54 OC1, plus full-sibling non-transgenic American controls). The data is still preliminary. For the potted 35S-OxO small stem assay done by Andy Newhouse's group, counting only those with obvious infections (disregarding no-takes), the height of the cankers was significantly different between Darling 58, Chinese, and non-transgenic American (n=36 Darling 58 OC1's, n=16 non-transgenic American, n=8 Chinese). Girdling also was significant after 29 days. ALL non-transgenic American stems were girdled, while NO Darling 58 stems were girdled. For Erik Carlson's potted Win 3.12 OxO (WX) inoculations, there was significant differences in terms of girdling. All non-transgenic controls wilted by 5 weeks post-inoculation, while NO transgenic WX events wilted (n=11 WX, n=7 non-transgenic controls).

USDA BRAG Program. The American Chestnut Research and Restoration Program at SUNY-ESF received a new grant from the USDA BRAG program (Biotechnology Risk Assessment Research Grants Program) to establish long-term restoration field trials. It is a collaboration with 5 PIs at SUNY-ESF and 2 PIs from 2 other universities. These include Bill Powell, Dylan Parry, Tom Horton, Colin Beier, and John Drake from SUNY-ESF, Sara Fitzsimmons from Pennsylvania, and Jason Holliday from Virginia. There will be two types of plots, one open field and one forested field located in three locations, NY, PA, and VA. The study will compare Darling American chestnuts to backcross, hybrids, and wild-type chestnuts.

Production. To increase the production of transgenic American chestnuts at SUNY-ESF, a field site was expanded by adding new fencing. The plot currently contains 50 transgenic American chestnuts and 50 mother trees with the intention of tripling the number of planted trees. The irrigation system also was updated to handle production.

Transformations. Currently the transgenic American chestnuts have a background genet of Ellis #1, a New York tree. To increase the diversity of the transgenics, new backgrounds will be transformed. One Northern genet will be chosen from the following: Alessi, Bass Mountain, Doc Stalter, Moss Lake, Spring Hole, or Zoar, all from New York, and one Southern genet will be used from the following: TG8A-1 from Virginia or AxW3-46B from Kentucky. Two separate transformations will be done to each genet chosen. One transformation will include the OxO with the 35S constitutive promoter, and one transformation will include the OxO with the Win 3.12 wound inducible promoter.

Regulatory Process. The documentation is almost complete to send to the USDA and is expected to be sent by end of September. This submission will be checked for completeness by the USDA and may not be the final version. The final form will be due six months after the USDA responds to the first proposal. We may not have to go through the EPA for regulation.

Mark Double, West Virginia University

B_3F_3 Planting at the University Forest, Morgantown, WV. Two hundred advanced backcross seedlings were planted in April/Sept 2015 at the University Forest near Coopers Rock in Preston County, WV. WVU forestry students and members of the Urban Forestry Club, helped establish the planting. An additional 100 backcross seedlings were planted in October 2016. In May 2018, 90 potted seedlings representing 4 lots, were used to replace dead seedlings. Also in May 2018, 17 American chestnuts from Allan Nichols in NY were planted at the site to start a mother tree orchard. Tree survival was assessed the first week of Aug 2018.

Lot	Number	% Alive	Planted	Type
1=D8-10-19	14	50%	11-Apr-15	Seed
2=D2-29-55	14	64%	11-Apr-15	Seed
3=D3-8-119	12	75%	11-Apr-15	Seed
4=D7-26-86	12	50%	11-Apr-15	Seed
5=D5-17-89	11	36%	11-Apr-15	Seed
6=D6-29-148	8	62%	9-Jun-15	Potted seedlings
7=D4-11-52	9	78%	9-Jun-15	Potted seedlings
8=W2-30-124	7	43%	9-Jun-15	Potted seedlings
9=D3-29-14	6	67%	9-Jun-15	Potted seedlings
10=W15-150	7	71%	9-Jun-15	Potted seedlings
11=W2-32-108	32	28%	11-Oct-16	Potted seedlings
12=W5-21-9	29	48%	11-Oct-16	Potted seedlings
13=W5-31-108	22	36%	11-Oct-16	Potted seedlings
14=D4-27-78	17	71%	11-Oct-16	Potted seedlings
Lot C	100	59%	5-Dec-15	Bareroot seedlings
15=D2-10-3	36	78%	10-May-18	Potted seedlings
16=D3-17-33	17	53%	10-May-18	Potted seedlings
17=W2-22-108	26	65%	10-May-18	Potted seedlings
18=W4-21-42	11	45%	10-May-18	Potted seedlings
American	17	82%	24-May-18	Potted seedlings

For seeds and seedlings planted in 2015, the average survival after three years was 55% and 61% for seeds and seedlings, respectively.

John Carlson, Pennsylvania State University

Penn State University Participants:

John Carlson, Director of the Schatz Center for Tree Molecular Genetics
Department of Ecosystem Science and Management
Pennsylvania State University
University Park, PA 16802
phone (814) 863-9164; fax (814) 865-3725; email jec16@psu.edu

Kim C. Steiner, Professor and Director of the Penn State Arboretum
Department of Ecosystem Science and Management
Pennsylvania State University
University Park, PA 16802

Phone (814) 865-9351; fax (814) 865-3725; email kcs@psu.edu

Charles Ray, Associate Professor, Research and Extension
Department of Ecosystem Science and Management
Pennsylvania State University
University Park, PA 16802
Phone (814) 865-0679; fax (814) 865-3725; email cdr14@psu.edu

Sara Fern Fitzsimmons, Regional Science Coordinator
Pennsylvania State University, and The American Chestnut Foundation
University Park, PA 16802
Phone (814) 863-7192; E-mail: sff3@psu.edu, sara.fitzsimmons@acf.org

UPDATES:

I. Chestnut Genome Sequencing Project Activities (**John Carlson**, Penn State)

Project team:

Penn State University - Charles Addo-Quaye, Nathaniel Cannon, Lynn Tomsho, Daniela Drautz, Lindsay Kasson, Tyler Wagner, Nicole Zembower, Abdelali Barakat, Richard Burhans, Webb Miller, Stephan Schuster, Tatyana Zhebentyayeva, Bert Abbott, and John Carlson
University of Tennessee - Margaret Staton, Nathan Henry, Jiali Yu, Matt Huff
University of Kentucky at Lexington – Shenghua Fan, Dana Nelson (and USFS), Bert Abbott
Virginia Tech University - Jason Holliday and Mihir Mandal
Texas A&M University - Nurul Islam-Faridi
The American Chestnut Foundation - Jared Westbrook, Sara Fitzsimmons, Fred Hebard (retired), Tom Kubisiak (and USFS; retired), Laura Georgi (retired).

Version 1 of the Chinese chestnut genome.

During the five-year USDA multi-state research project NE-1333, the first version of the Chinese chestnut genome was sequenced and assembled and released to the public in January 2014 at the website https://hardwoodgenomics.org/chinese-chestnut-genome, developed and curated by Margaret Staton at the University of Tennessee - Knoxville. The version 1.1 genome assembly was produced for the TACF cultivar Vanuxem. It comprised 724.4 Mb in 41,270 scaffolds, averaging app. 40,000 bp in length. This represents at least 91% of the Chinese chestnut genome, based on estimates from pre-sequencing era studies. A total of 36,146 likely genes were identified. In addition, DNA clones physically spanning the 3 blight resistance QTL were separately sequenced and assembled into a total of 395 scaffolds covering 13.8 Mb. Over 1,900 genes were found in the QTLs, including 194 known stress-response genes, from which 15 candidate genes for blight resistance were selected for further study. The genome browser and the QTL browser website has had thousands of visits from across the globe, for gene searches and scaffold, gene, transcripts and predicted protein data downloads.

The version 1.1 Chinese chestnut genome was used in various studies which demonstrated the power provided by a genome reference, including genotyping and assessing genetic variation among accessions, species, and breeding material in the CAES and TACF orchards. For example, in one study, relatively shallow (10X) depth of DNA sequence data was produced for one *C. alnifolia* genotype, one *C. crenata* genotype, five *C. dentata* genotypes, one *C. henryii* genotype (Chinese chinkapin), four *C. mollissima* genotypes, one *C. ozarkensis*

genotype, one *C. sativa* genotype, one *C. seguinii* genotype, three third backcross hybrids from the TACF breeding program, and the BC3 C. dentata x C. mollissima parental genotypes - B3119 and B3176. Alignment of the parental and BC3 genotype genome sequences to the Vanuxem reference genome clearly revealed a separation of Chinese, American and the intermediate F1 hybrid genomes, as well as the transitional nature of BC3 genomes. The study also suggested that the TACF backcrossing program's goal of converting the donor genome to a mostly American genome content is still a work in progress. These results were presented to the TACF at their annual meeting in October 2015, held at Penn State University conference center.

In another important study recently published by LaBonte, et al. (2018), our version 1.1 genome was used as the reference to detect signatures of selection in the Chinese Chestnut genome which predicted genes potentially related to flower phenology and development, fruit maturation, and secondary metabolism, and included some genes homologous to domestication genes in other woody plants. These candidate genes may prove useful in chestnut improvement,

Improved version of the Chinese chestnut genome.

The quality and contiguity of the version 1.1 genome for Chinese chestnut is on par with most previously published draft plant genomes. It is entirely sufficient for studies in genetic diversity, in analysis of gene expression, for gene function characterization, for DNA marker generation, and for most phylogenetic and gene evolution analyses. However, for application of the chestnut reference genome in Genome-Wide-Selection to advance back-cross breeding and disease resistance introgression programs, such as TACF is conducting, chromosome-scale sequences assemblies of scaffolds are required. We thus worked for the past four years on producing an improved 'version 2' of the Chinese chestnut genome with proper gene order and approximates length at the chromosome-scale. The available approach to accomplish this involved bridging and merging scaffolds using longer genome sequences followed by anchoring large scaffolds to positions on genetic linkage maps using DNA marker sequences. Taking this approach, we first reduced the number of scaffolds to 14,358, covering 784Mb of genome sequence (app. 98% of the estimated genome size) based on BAC-end sequences distributed across the physical length of the genome (Kubisiak et al, 2013b). The 5,745 largest scaffolds were anchored to the integrated genetic-physical map for Chinese chestnut (Kubisiak et al, 2013a), producing a set of 12 "pseudo-chromosome" sequences. However, the Vanuxem pseudo-chromosome sequence assemblies were not highly consistent with the order of loci on several, high-density genetic linkage maps produced by Tatyana Zhebentyayeva for BC3F2 families. This may have resulted from the relatively low number of Vanuxem-parent loci (590) that we had to work with in the Vanuxem cv. ♀ × Nanking cv. ♂ (V × N)] genetic map (Kubisiak et al, 2013b). Thus, we decided to try building pseudo-chromosomes again, using higher density Vanuxem maps.

In the past year, funding from a USDA NIFA grant with TACF allowed us to generate very long genome sequences by the PACBio technology for bridging scaffolds and closing gaps, reducing the number of genome scaffolds to 12,684, covering 784 Mb (~98% of genome). New pseudo-chromosome sequences were assembled by anchoring 4,314 of the scaffolds to DNA markers in Shenghua Fan's genetic map for Vanuxem. We also produced 8 Gbases of new RNA sequence data from tissues of the Vanuxem reference genotype to aid Margaret Staton's group

in finding and annotating genes in the new pseudo-chromosomes. This new assembly, referred to as Version 3.0, will be released to the public soon via a new genome browser at the Hardwood Genomics website and a peer-reviewed publication, which will include gene positions, where RNAs have been mapped, and organization relative to other chestnut maps and tree genomes.

During the USDA-funded project, Jason Holiday at Virginia Tech and Jared Westbrook at TACF developed a draft Genome-Wide-Selection model for use in accelerating the TACF breeding program, using the 14,358-scaffold assembly. The Staton group produced a set of potentially diagnostic 714,039 SNPs from sequences of three American genotypes for use in developing the GWS model(s). The GWS model can now be refined further and tested using the new Vanuxem Version 3 genome assembly pseudo-chromosomes.

II. Breeding and field trials (Kim Steiner, Penn State University**):**
Project team:
Penn State University - Kim Steiner;
The American Chestnut Foundation – Sara Fern Fitzsimmons and Steve Hoy.

Dr. Steiner has a long-time partnership with The American Chestnut Foundation on breeding for blight-resistance in American chestnut and on research towards restoring the species to Appalachian forests. TACF's Northcentral Regional Breeding Coordinator is based in the Steiner lab, and the Pennsylvania TACF Chapter's statewide and regional breeding programs have been coordinated by Steiner's PhD student S.F. Fitzsimmons. Dr. Steiner and Sara Fitzsimmons have established a large field trial, on the Penn State campus, of many families of TACF 3rd back-cross generation progeny continue to being evaluated for blight resistance and form. Dr. Steiner has also provided oversight to TACF breeding, restoration, and research efforts as Chair of the Science Cabinet from 2007 to 2012, and currently as Chair of the TACF Board of Directors.

III. Wood phenotyping and extension (Charles Ray, Penn State University):

Penn State University started a wood sample collection in 1909 with H. J. Heltman's collection of specimens from 48 tree species on the Mont Alto campus. In 1956, wood importer Joseph Stearns made a major donation of wood specimens (reportedly between 2,400 and 2,500). To accommodate the larger collection, the then-School of Forest Resources acquired a large cabinet with hundreds of drawers to hold the specimens. Wood technology professor Newell Norton documented part of the 32 different donated collections within the Penn State collection. Dr. Ray and his staff are taking high-resolution, magnified photos of the app. 18,000 specimens in the current "Xylarium" (wood collection), with the intent to make the photos available online, and to make the collection easily accessible to researchers studying the genetics and molecular properties of wood. This collection includes chestnut wood samples from various sources, which is already serving as a reference for researchers and TACF members. We hope that the chestnut wood collection will be supplemented with additional material from NE colleagues and other chestnut enthusiasts.

IV. Outreach (Carlson and Steiner teams, Penn State University**)**

The Schatz Center for Tree Molecular Genetics hosted the 2018 annual meeting of the USDA multi-state research project NE-1333, held in the historic Nittany Lion Inn on the Penn State University Park campus, September 6 to 9, 2018. This was the 36th Annual Meeting of this USDA multistate research project, and the last gathering as project number NE-1333. There were 20 excellent talks presented, providing Agriculture Experiment Station reports and research updates on a wide range of topics, from genome sequencing to fungal sleuthing to super donors. Fifty people attended the meeting, representing a wide diversity of experience and affiliations, and including undergraduate students, graduate students, post-doctoral fellows, visiting scientists from abroad, new professors, old professors, and TACF members.

The meeting also included a field tour guided by Sara Fitzsimmons, Steve Hoy, and Kim Steiner, with stops at 2) their TACF BC3-F2 progeny trial in the Arboretum, 1) Greenhouse results from small stem inoculation assays of BC3-F3 individuals, and 3) a B2F2 field trial established in 1997 at a protected PA State Gamelands site that is still being monitored.

The final session was the annual business meeting which was opened by our Administrative Adviser Bradley Hillman at Rutgers University, who provide an overview of the group's 36 year history, and well-wishes for the new version of the USDA multistate chestnut research project - NE-1833. Dr. Hillman also presented a plaque to the out-going secretary, Mark Double, in recognition of his extraordinary dedication and the key role that he played over many years in the project. Finally new officers were elected for the 2018-2019 year (Chair- Matthew Kasson, West Virginia University; Vice-chair- Andrew Jarosz, Michigan State University; and Secretary- Laura Barth, TACF).

PLANS FOR THE COMING YEAR:

Work in the coming year will focus on:

1) Assist the secretary and chair in the process of initiating NE-1833
2) Finalize the Chinese chestnut chromosome-scale genome assembly.
3) Test new approach for genome assembly based on 'chromatin interaction data', to go from short-read de novo assemblies directly to chromosome-scale sequences.
4) Submit refereed journal article on Chinese chestnut reference genome (in preparation).

Dorothy Tchatchoua, Pennsylvania State University (Fulbright Scholar from the Cameroon)

Breeding strategy of genetic stocks of *Castanea sativa* in Greece.

- Chestnut in Greece exist under three management levels: old-growth natural, coppice natural and grafted populations.
- High variation was observed in both genetic and quantitative traits in chestnut populations in Greece.
- Considerable variation was found on growth traits using juvenile material from six European chestnut populations
- There is the presence of genetic variation between six extreme chestnut European populations on adaptive traits.

- Studies on eighty-two European chestnut populations using 73 ISSR and 16 isoemzyme loci reported large possibilities of outcoming gene flow to maintain diversity in these populations.

Reasons for breeding strategy of chestnut. There exist variability in morphological, nuts and adaptability in chestnut and thus the possibilities to develop a breeding strategy in the chestnut populations in Greece. This will permit the identification of superior genotypes by their phenotypes and multiple them via micropropagation techniques.

Her objectives were:

- Evaluation of field trials established in Taxiarchis (in the Macedonia region of Greece)
- Propagation of the genetic stock of *C. sativa*

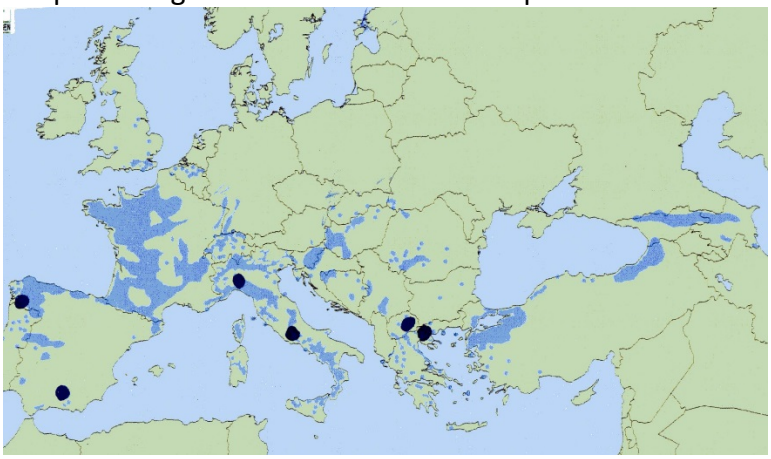
Specific objectives were:

- Investigate genetic variation among and within provenances on growth traits
- Estimate quantitative genetic parameters in growth traits
- Propagate plus - trees via tissue culture

Materials and Methods

- The genetic material used in the field trials consisted of:
  - 143 open pollinated families
  - Six provenances
  - Two provenances from Spain, Italy and Greece respectively

Map showing countries and location of provenances is as follows:



Seeds were sown during winter 2001 and planted in March-April 2002. A single tree plot with one seedling per family was utilized. A complete randomized block design was implemented with 20 replicates. Spacing was 3 x 3 m apart.

Data, listed below, as collected in June 2005, 2006 and 2007.

Survival

Height

Diameter

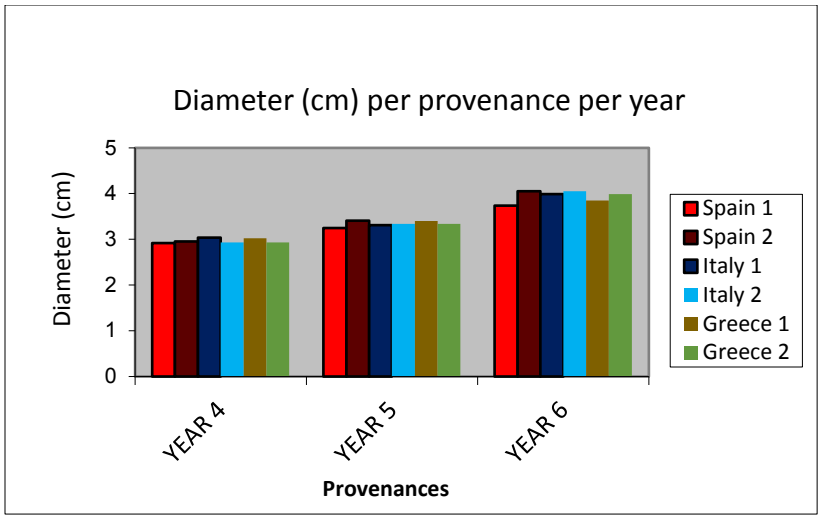
Volume index calculated as (π x height x [diamter^2]/4) cm^3

Number of leaves

Number of shoots

Shoots with diameter ≥1.5 cm

Provenance means comparison (diameter) is shown in the following graph.



- Provenances changed position across the ages.
- EU-37 (Sicilia, Italy 1) at the top at age four while EU-11 (A. Coruna, Spain 2) occupies regularly the first position at ages five and six.
- Among provenances for diameter were significant at 5% level for most years.

Family means comparison

- The variation in family tree diameter range from 1 to 7 cm. Family mean range from 2.6 to 5.4 cm.
- The family height range from 20 – 200 cm. Family means range from 67.7 to 170.7 cm at all ages.
- The range between family volume indexes was 21.9 to 4235.5 cm^3. Family means range from 468.3 to 3084 cm^3 at all ages.
- The variation between family no. of leaves in the data ranged from 20 to 1550. Family means varied from 175.0 to 1075 at all ages.
- Families Spain 2-60, Italy 1-30, Italy 1-35 and Italy 1-18 and Greece 1-2 appeared in three of the traits among the best 10 families.
- Families Spain 2-60, Spain 2-45, Greece 1-40, Italy 1-35, Italy 2-37, and Italy 2-31 occurred in all years among the 10 best families within traits.

Conclusions:

- From the analysis it can be noted that there is considerable variation among and within provenances in the Taxiarchis test site.
- Variation among provenances in growth traits followed geographical pattern with EU-11 (Coruna, Spain 2) and EU-56 (Pellice, Italy 2) from the wet region being the best provenances for growth traits.
- There was stability among provenances for growth traits, with EU-11 (A. Coruna, Spain 2) being best for diameter, EU-56 (Pellice, Italy 2) for height and Volume index while EU-7 (Malaga, Spain 1) was best for number of leaves
- The average rate of mortality was low 2.5% only
- Growth traits are under strong genetic control.
- Sufficient heritability was recorded to permit selection.

- Genetic improvement using the combined selection method might be achieved more quickly.
- Selection will favour a large tree volume index production but due to the large error variance, selection for height should be much more preferable.
- There are considerable scope for rapid improvement through multiple trait selection

Tetyana Zhebentyayeva, Penn State University

Genetic mapping the resistance/susceptibility response in chestnut. PRR resistance mapping has shown 17 genome regions (QTLs) controlling resistance in 5 backcross families involving two Chinese chestnut grandparents ('Mahogany' and 'Nanking'). The effects of these regions varied, suggesting the presence of both major and minor effect genes. Regions with large effects were found on Linkage Group E (LG_E) in families with both Chinese grandparents and on LG_K in families with 'Mahogany' grandparent. Regions with smaller effects were found on LG_A and LG_C. A manuscript in in preparation.

Objective 2. ***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***

Mark Double, West Virginia University

NE-140 Pathogenicity Study from 1985. William MacDonald, Mark Double (WVU), Jack Elliston (deceased) (Conn. Ag. Exp. Station), Gary Griffin, Martha Roane (deceased) (Virginia Tech), Dennis Fulbright (Michigan State University) and William Stambaugh (Duke).

In the early 1980s, research groups working with *C. parasitica* isolates used their own virulent standard. The purpose of this study was to compare the pathogenicity of eight *C. parasitica* isolates in virulence tests conducted by various institutions. An 8X8 Latin Square design was used and American chestnuts were inoculated in three states (Connecticut, West Virginia and North Carolina). Eight uninfected trees were selected at each site and inoculated with the following isolates:

- Ep 155 (CT)
- WK (Virginia Tech)
- Ep 523 (CT)
- Ep 146 (WV)
- SOSM (Duke)
- CR (Virginia Tech)
- CL1-BLBO (Michigan State University)
- CL1-16 (Michigan State University)

Trees were inoculated in July 1984 and measured in July 1985. Jack Elliston developed a virulence quantification (*sensu lato*): canker area + number of stromata/10 + percentage of stromata with perithecia/10.

Data, as follows, were only taken at sites in CT and WV.

WV inoculations:		**CT inoculations:**	
Virulence	***Isolate***	***Virulence***	***Isolate***
838.1 A	Ep 155	1190.1 A	SOSM
704.1 A	WK	1127.4 AB	Ep 155
689.6 A	Ep 523	946.8 BC	CR
658.1 A	EP 146	889.9 CD	Ep 523
593.5 A	SOSM	865.6 CD	WK
294.4 B	CR	838.4 CD	Ep 146
256.8 B	CL1-16BLBO	690.8 D	CL1-16BLBO
208.5 B	CL1-16	454.3 E	CL1-16

Backcross orchard for assessment of host resistance combined with hypovirulence. (in cooperation with Fred Hebard and Sara Fitzsimmons, The American Chestnut Foundation)

Six replicate plots, each containing 150 trees, have been established at the Plant and Soil Sciences Farm in Morgantown, WV to assess the interaction of host resistance and virulent/ hypovirulent strains of *Cryphonectria parasitica*. The planting was established in 2006 and dead trees were replaced annually from 2007-2011. In three plots, naturally occurring cankers were treated with hypovirulent isolates; three plots were not inoculated. During the first five years, many of the blight susceptible trees (American, Europeans and some backcross) died. In July 2013, trees over 3 cm were inoculated with the WK strain to assess their blight resistance/susceptibility. Beginning in 2015, cankers in the three Hv-treated plots were treated with four Hv isolates (WK Hv, COLI, GH2, Euro7). In 2017, a fourth Hv isolate (SR 136-3) was added so all known *vic* genotypes were included in the treatment inoculum.

Hv Isolates	***vic* Genotype**
Weekly Hv	2211-11
COLI	1122-11
GH2	1211-11
Euro 7	2111-11
SR 136-3 Hv	2212-22

The data used for comparisons reflects only trees that were alive in 2015 when the Hv strains were first introduced (165 trees were not included). Data in the following table are from July 2018.

	Non-Treated Plots		**Treated Plots**	
Species/BC	**Total**	**Alive**	**Total**	**Alive**
American	72	57%	77	79%
B_2F_2	5	60%	15	100%
B_2F_3	88	86%	93	86%
B_3F_2	48	73%	49	84%
Chinese	93	96%	87	94%
European	44	25%	63	64%

The benefit of hypovirus canker treatment to maintain trees is demonstrated, especially for American, European and B_2F_2 trees.

Laurel Rodgers, Shenandoah University

Comparison of the fungal microbiome in chestnut trees resistant to *C. parasitica* to those that are not resistant to *C. parasitica*.

Summary. The long-term goal of this project is to determine if there are different fungal endophyte communities living in chestnut trees resistant to *Cryphonectria parasitica* (Chinese chestnuts and chestnut hybrids) compared to chestnut trees that are not resistant to *C. parasitica* (American chestnuts). Endophytes are fungi that grow within a plant without causing harm and likely prevent colonization of pathogenic fungi. Endophytes that provide resistance against *C. parsistica* can be used as a biocontrol against the fungus, assisting in the reestablishment of the American chestnuts into our forests. We have collected samples from American chestnut, Chinese chestnut, and hybrid trees at two different locations are currently identifying fungi growing within these trees using traditional Sanger sequencing. Once identification is complete, we will not only be able to compare the fungal microbiomes of different chestnut species, but we also will be able to compare the fungal communities from two different research sites.

Introduction. Endophytes are defined as fungi and bacteria living within a plant without causing harm. Surveys within the last thirty years have found a surprising number of fungi growing in healthy trees. For example, an average of 11.5 fungal species were isolated from leaves and twigs of the American hornbeam (*Carpinus caroliniana)* located in New Jersey and West Virginia. In another study, Danti *et al* (2002) isolated forty-four different fungal species from ten European birch (*Fagus sylvatica*) trees growing within a 200m-diameter area. Despite the fact that fungi have been found in living tissue of all plants surveyed to date, fungal endophytes represent a significantly understudied, and poorly understood, component of our ecosystem.

Like the bacterial microbiome in the human body, endophytes in a tree are likely to play a significant role in protection from pathogens. For example, fungal endophytes may outcompete pathogenic fungi within a tree. Arnold *et al* (2003) investigated the leaf mortality of *Theobroma cacao* seedlings after being inoculated with six *Theobroma cacoa* endophytes and then challenged with a pathogenic fungus. The seedlings inoculated with endophytes had a significantly lower rate of leaf mortality compared to control seedlings that were not inoculated prior to the administration of the pathogenic fungus. While some endophytes do stimulate systemic defenses within a plant, it is likely in this case that the introduced endophytes were inhibiting the pathogen through direct interactions.

Several endophytes are also known to secrete anti-microbial chemicals, which are likely to inhibit the growth of pathogenic fungi. *Acremonium sp.* naturally grows on the European yew (*Taxus baccata)* and inhibits the growth of fungi in *in vitro* studies. *Acremodium sp.* produces the anticancer and antifungal peptide leucinostatin A. Unlike many plants, the yew tree has no adverse reaction to the presence of leucinostatin A. Based on their study, Strobel and Hess *et al* (1997) hypothesize that *Acremodium sp.* protects the yew tree from colonization by pathogenic fungi. In another example, *Muscodaor albus* produces a combination of volatile

antimicrobial compounds that inhibit the growth of other fungi on the cinnamon tree (*Cinnamomum zelanicum*). Improving our understanding of natural endophyte populations and how they may vary between tree species and between geographical locations will be valuable when combating the spread of devastating diseases throughout our forests.

The American chestnut (*Castanea dentata*) tree is just one species that may benefit from the enhancement of natural endophyte populations. Prior to the early 1900s, the American chestnut was the dominant tree species within eastcoast US forests. The tree was a keystone species both economically and environmentally. Unlike the dominant species today, the oak and the hickory, the American chestnut provided a reliable supply of nuts every year. The high tannin content within these trees also supported a large leather tanning industry and its wood was highly valued for timber because it resists decay without the chemical treatment needed for today's common timber trees. At the turn of the 20th century, the fungus *Cryphonectria parasitica* was accidently introduced to the United States. Unlike the Chinese chestnut (*Castanea mollissima*), the American chestnut has no resistance to *C. parasitica*. By the middle of the 20th century, nearly all American chestnuts had been wiped out from our forests.

The goal of this project is to compare the normal fungal populations growing in the American chestnut to those in the Chinese chestnut and chestnut hybrid trees that are resistant to *C. parasitica* in order to identify endophytes that may contribute to resistance. Due to chemical differences within each tree, it is reasonable to hypothesize that different fungal species inhabit each species. These differences will provide further clues that indicate why the Chinese chestnut tree is more resistant than the American chestnut. This study will allow us to identify an endophyte, or a group of endophytes, that grow on resistant chestnut trees, but not in American chestnut trees lacking resistance. These endophytes can then be assessed for use in the development of biocontrol methods against *C. parasitica* in future American chestnut reforestation projects.

Summary of Methods. In June 2018, we collaborated with The American Chestnut Foundation (TACF) and visited two orchards: Mount Zion in Aldie, Virginia and The Ranch in Culpeper, Virginia. We collected bark plugs from American, Chinese, hybrid, and F1 chestnut trees. F1 chestnut trees were only present at The Ranch. TACF was at both locations inoculating the trees with two strains of *C. parasitica.* We collected bark plugs into sterile 1.5mL tubes as they were removed from the trunk of trees sterilized with 70% isopropanol. After isolation, each plug was transport in a cooler with ice to the lab at Shenandoah University and stored overnight at 4 degrees Celsius.

The following day, each plug was placed in nutrient free, 1.5% agar plates and observed daily for a week. Hyphae growing from the samples were removed and placed on 3.9% potato dextrose agar (PDA) plates. Each fungal sample was grown for 3-5 days and then transferred to a new plate in order to confirm a pure sample was present on the agar plate. Prior to DNA isolation, hyphae were transferred onto sterile cellophane on PDA plates and incubated for 2-3 days. DNA was isolated from hyphae using a Qiagen DNeasy plant mini kit (Qiagen, Hildon, Germany). The fungal ITS region, the DNA region between the large and small ribosomal subunit genes, was then amplified by PCR using ITS 1 (TCCGTAGGTGAACCTGCGG) and ITS 4 primers (TCCTCCGCTTATTGATATGC). PCR samples were cleaned using a QIAquick® PCR Purification Kit (Qiagen, Hildon, Germany) and submitted to Euruofins Genomics (Louisville, KY)

for Sanger sequencing. The sequences were aligned using CodonCode Aligner and fungal identity for each sequence was determined using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the UNITE database (https://unite.ut.ee/).

Results. We sampled a total of 45 trees from each orchard, pulling two plugs from each for a total of 180 bark plugs. A combination of American, Chinese, and hybrid trees were collected from each orchard, with the addition of F1 trees from The Ranch (Table 1).

Table 1. Number of plugs collected from sample sites

Location	American	Chinese	Hybrid	F1	# Trees Sampled	# Bark Plugs
The Ranch	10	10	15	10	45	90
Mt. Zion	4	8	33	n/a	45	90
					90	180

A total of 361 fungi samples were isolated from all plugs collected. From the Ranch, 39 fungi samples from Chinese, 34 from American, 46 from hybrids, and 39 from F1 chestnut trees were isolated. At Mount Zion, 39 fungi from Chinese, 20 from American, and 144 from hybrid chestnut trees were isolated (Table 2). So far, about ten samples from the Ranch and thirty from Mount Zion have been sequenced and identified, but approximately 100 from the Ranch and 150 from Mount Zion still need to be sequenced.

Table 2. Number of fungi isolated

Location	Chinese	American	Hybrid	F1	Total
The Ranch	39	34	46	39	158
Mt. Zion	39	20	144	n/a	203
					361

Of the species identified thus far at Mount Zion, a majority of the fungi are parasitic fungi. However, based on brief literature searches, three species of fungi, *Fimetariella rabenhorstii, Hypoxlon submonticulosum, and Albifimbria verrucaria* isolated from hybrid chestnut trees could be potential endophytes (Table 3). We were surprised to find the fungus *Gnomoniopsis smithogilvyi* growing within one of our hybrid trees. This is a fungus known to cause cankers on chestnuts in Europe. A more extensive study will need to be completed in order to determine how wide spread *G. smithogilvyi* is within the US and whether it will act as a pathogen in the American chestnut tree.

Table 3. Fungi species identified thus far at Mt. Zion (potential endophytes highlighted in blue)

American Chestnut	Chinese Chestnut	Hybrid trees
Biscogniauxia mediterranea	*Biscogniauxia mediterranea*	*Biscogniauxia mediterranea*
	Biscogniauxia atropunctata	*Biscogniauxia atropunctata*
Coniochaeta	*Coniochaeta*	*Gnomoniopsis smithogilvyi*
	Diplodia seriata	*Diplodia corticola*

		Botryosphaeria dothidea
		Fimetariella rabenhorstii
		Hypoxylon submonticulosum
		Xylariales
		Albifimbria verrucaria

At this point, sufficient sample have not been identified to make any conclusions about the data. We will continue sequencing samples over the next year and present our findings and any conclusions at the next meeting.

Bailey Hamilton and Teresa Zielinski, Shenandoah University

Identifying fungi growing inside of chestnut nuts. In addition to the project described in detail by Laurel Rodgers, an investigation began looking at the fungi present inside of American and Chinese chestnut nuts. Chestnut burs were collected from the ground under an American chestnut at the Blandy Research center in Boyce, VA and the ground under two Chinese chestnut trees near Lovingston, VA. Burs also were collected from the ground and on an American chestnut tree located at Lesesne State Forest in Roseland, VA. Once in the lab, seeds were soaked for one minute in 70% ethanol, then for ten minutes in 10% bleach + tween 20, and finally soaked for one minute in sterile water. After sterilization, seeds were cut open with a sterile knife. Pieces of the case and pieces of the nut were placed onto 1.5% nutrient agar. The remaining procedures for isolating, extracting DNA, sequencing, and identifying each fungus sample was identical to the methods described by Rodgers.

All samples collected have not been identified, but those that have been are listed below in Table 1. Fungi were found growing in all nuts sampled, both on the inside of the case and within the nut tissue. At this time, we have identified one sample of *G. smithogilvyi* within an American chestnut nut. We will need to identify our remaining samples before we can compare the fungi growing in the nuts of each tree species.

Table 1: Fungi species identified thus far in American and Chestnut tree nuts

American, Ground, Blandy	**American, Ground, Lesesne**	**American, Tree, Lesesne**	**Chinese, Ground**
Mucor fragilis	*Fusarium proliferatum*	*Penicillium*	*Fusarium* (various species)
Many not yet identified	*Mucor fragilis*	Many not yet identified	*Gibberella thapsin*
	Cytospora genus		*Penicillium*
	Gnomoniopsis smithogilvyi		*Irpex lacteus*
	Penicillium		*Bipolaris* (species not clear)
	Biscogniauxia mediterranea		*Schizophyllum commune*
	Quambalaria cyanescens		*Alternaria* (various species)

Matt Kasson, West Virginia University

Two-year study of hypovirus transmission by engineered super donor strains into a vegetative incompatibly diverse natural population of the chestnut blight fungus *Cryphonectria parasitica*. D.L. Nuss, W.L. MacDonald, M.T. Kasson, C.M. Stauder, A.M. Metheny, M.L. Double. The development of strains of *Cryphonectria parasitica* with enhanced ability for strain-to-strain transmission of virulence-attenuating hypoviruses was reported by Zhang and Nuss (2016, PNAS 113:2062-2027). This was accomplished by systematic disruption of genes that regulate the vegetative incompatibility (*vic*) fungal allorecognition system to remove restrictions to mycovirus transmission. The results of laboratory transmission studies predict that the SD formulation could circumvent *vic* imposed restrictions to virus transmission by serving as an effective vector to introduce hypovirus into field strains representing all possible *vic* genotypic combinations of the six defined diallelic *vic* genetic loci. We report the results of a two-year field study to test this prediction in a forest setting.

Cryphonectria parasitica strains used in this study included *vic* genotype tester strains (Cortesi and Milgroom, 1998) EU5 (ATCC MYA-1048) and EU6 (ATCC MYA-1049) and the super mycovirus donor strains SD328 and SD82. The convention used to describe the *vic* genotype of *C. parasitica* strains specifies which allele, designated 1 or 2, is present at the six defined diallelic *vic* genetic loci. For example, the *vic* genotype for the *C. parasitica* reference strain EP155 is *vic1-2*, *vic2-2*, *vic3-1*, *vic4-1*, *vic6-2* and *vic7-2* (abbreviated 2211-22). The *vic* genotypes for strains EU5 and EU6 are 2211-22 and 2111-22, respectively, while the *vic* genotypes for SD strains 328 and 82 are ~~2~~2~~1~~1-~~22~~ and-~~2~~1~~1~~1-~~22~~ (strikes indicate a gene disruption at the respective *vic* loci). For this study, the EU and SD strains were infected with hypovirus CHV-1/EP713.

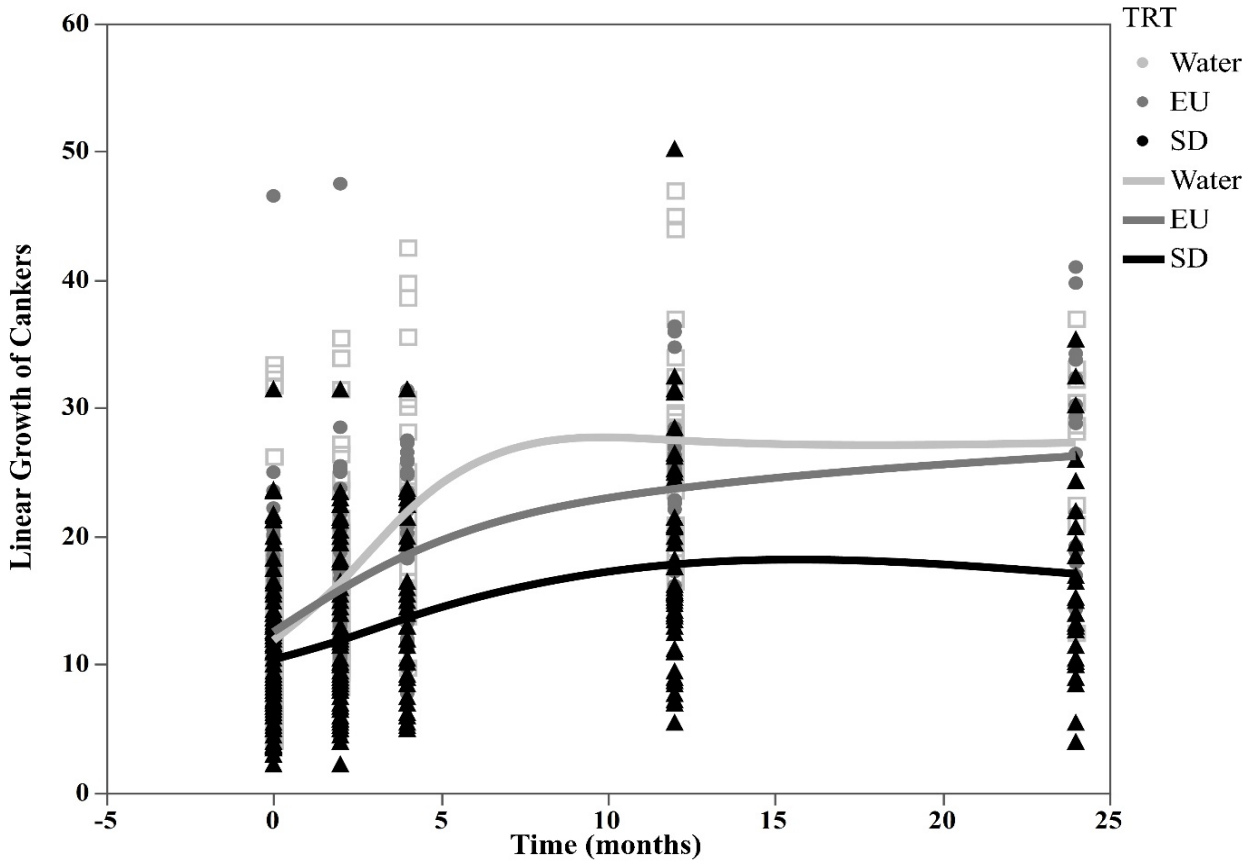
Three plots were established in the Savage River State Forest near Grantsville, MD in a clearcut stand containing an abundance of infected American chestnut (*Castanea dentata*) root sprouts symptomatic for chestnut blight. American chestnut stems with up to three *Cryphonectria parasitica* cankers, each being less than 75% of the stem's circumference, were selected in each plot. The following number of trees and cankers were included for each plot: SD Plot: 19 trees/41 cankers; EU Plot: 17 trees/33 cankers; and Water Agar Plot: 18 trees/31 cankers.

Each treatment was randomly assigned to one of three designated plots. Cankers in the SD plot were treated with a combination of CHV-1/EP713-infected strains SD 328 and SD 82. EU5/6 plot cankers were treated with the combination of CHV-1/EP713-infected strains EU5 and EU6 that have the same *vic* genotype as SD328 and SD82 without the gene disruptions. Water agar plot cankers received treatments with an agar slurry without any fungal inoculum to serve as a negative control.

vic genotyping. A multi-locus PRR protocol (Short el. al 2015, AEM 81:5736-5742) was used to assess the *vic* genotype of virulent *C. parasitica* isolates from pre- and post-treatment cankers and hypovirulent isolates from post-treated cankers. Pre-treatment vic genotyping revealed a high level of diversity (34 of 64 known possible genotypes). The percentages of treated cankers from which Hv isolates were recovered for SD and EU plots were 94.2% (65 of

69) and 48.9% (24 of 49), respectively, indicating enhanced hypovirus transmission by the SD formulation.

Canker linear growth. Cankers were measured and subjected to a one-time treatment in July 2016. New cankers were identified in 2016 (Sept and Nov), 2017 (May, July, Sep and Nov) and 2018 (June). At each visit, cankers were measured, sampled and treated with the plot-appropriate inoculum. Two-year linear growth (L+W)/2 of cankers in all three treatment plots is presented in the following figure. SD cankers are significantly smaller than those treated with EU and WA after two years, demonstrating efficient hypovirus transmission by engineered SD strains into a vegetatively incompatible diverse natural population of the chestnut blight fungus.



Amy Metheny, West Virginia University

Treatment delivery of superdonor fungus. A.M. Metheny, D.L. Nuss, M.T. Kasson and W.L. MacDonald

The purpose of this project is to assess how treatment type and hypovirus can impact efficacy of the superdonor strain of *Cryphonectria parasitica* when used to preserve the longevity of American chestnut stems. Fifty trees were selected, most were free of cankers, at a site in the Savage River State Forest near Grantsville, MD. For those trees completely free of infection, three artificial cankers were created, at 50 cm, 100 cm and 150 cm with a virulent isolate (EU 12). For those trees with one natural canker, only two artificial cankers were established, resulting in 161 cankers in the study, 29 are natural and 131 are artificial. The

natural infections were sampled and will be genotyped. Cankers were initiated on 22 June 2017 and by 8 August 2017, the average canker size was 6 cm (length) by 4 cm (width). The three treatments included: (1) scratch (a beehive comb was used to scratch through the bark of the canker to the cambium layer and inoculum spread over the wounds); (2) punch (a leather punch was used to create circular wounds around the canker margins and interior which are then filled with inoculum); and, (3) paint (inoculum was spread on the canker using a paintbrush without wounding the stem). The efficacy of two CHV-1 hypoviruses, Euro 7 and Ep 713, also will be compared. Inoculum was comprised of a mixture of either SD 328 (713) and SD 82 (713) or SD 328 (Euro 7) and SD 82 (Euro 7). All cankers on a tree were treated with the same treatment method and hypovirus. Trees were selected randomly for each treatment. Cankers were measured (length and width) every three months until August 2018 (one-year anniversary) at which they were sampled and bark samples were cultured to assess hypovirus transmission. Resulting isolates will genotyped according to the method of Short et al. (App. Env. Micro. 2015. 81:5736-5742) to assess *vic* genotypes of virulent and hypovirulent isolates. By August 2018, the number of cankers increased from 161 to 217, including a total of 86 natural cankers. Preliminary results indicate that cankers treated with the Euro 7 hypovirus applied by scratch and punch had less growth over the 1-year period when compared to the paint treatment. Cankers treated with the Euro 7 hypovirus grew slower overall when compared to cankers treated with the EP 713 hypovirus across all treatments. Wounding appears to be essential for efficient hypovirus transmission.

Andrew Jarosz, Dennis Fulbright (emeritus) and Matt Kolp, Michigan State University

Michigan chestnut census. The 23rd census of five American chestnut populations was completed in August 2018. Seed production was above average at the three recovering populations. In contrast, new seedling occurrence was extremely low at all populations except County Line. No seedlings were found at the two epidemic sites, Leelanau and Missaukee. Indeed, no new seedlings have been found at Missaukee since 2013. Large trees are dying back rapidly at both epidemic sites. There is only one tree at Missaukee with a diameter at breast height (DBH) > 10cm. Large trees are doing well at the Roscommon and County Line recovering sites, while trees continue to decline at the third recovering site, Frankfort. Although, Frankfort has long been considered a recovering site, that status is now in doubt. Hypovirus prevalence has been declining, and in consequence, trees at Frankfort have been declining slowly for almost a decade.

Intra-canker variability for secondary fungi. (In cooperation with Bill MacDonald and Mark Double, West Virginia University). This topic was the thesis project of Matt Kolp who graduated in April 2018. The fungal community of chestnut blight cankers in surviving chestnut populations in northern Michigan and in West Salem, Wisconsin contains virulent (CP) and hypovirulent *C. parasitica* (HCP) along with dozens of other fungal taxa (collectively Non-CP). Our prediction that canker communities would be highly structured spatially (Figure 1a & b;) was totally incorrect. Instead, cankers appear to be mosaics with CP, HCP and Non-CP being intermixed throughout the canker (Figure 1 c & d). Sampling 201 cankers for up to five years, found that the average canker is 33.2% CP, 33.0% HCP and 33.8% Non-CP.

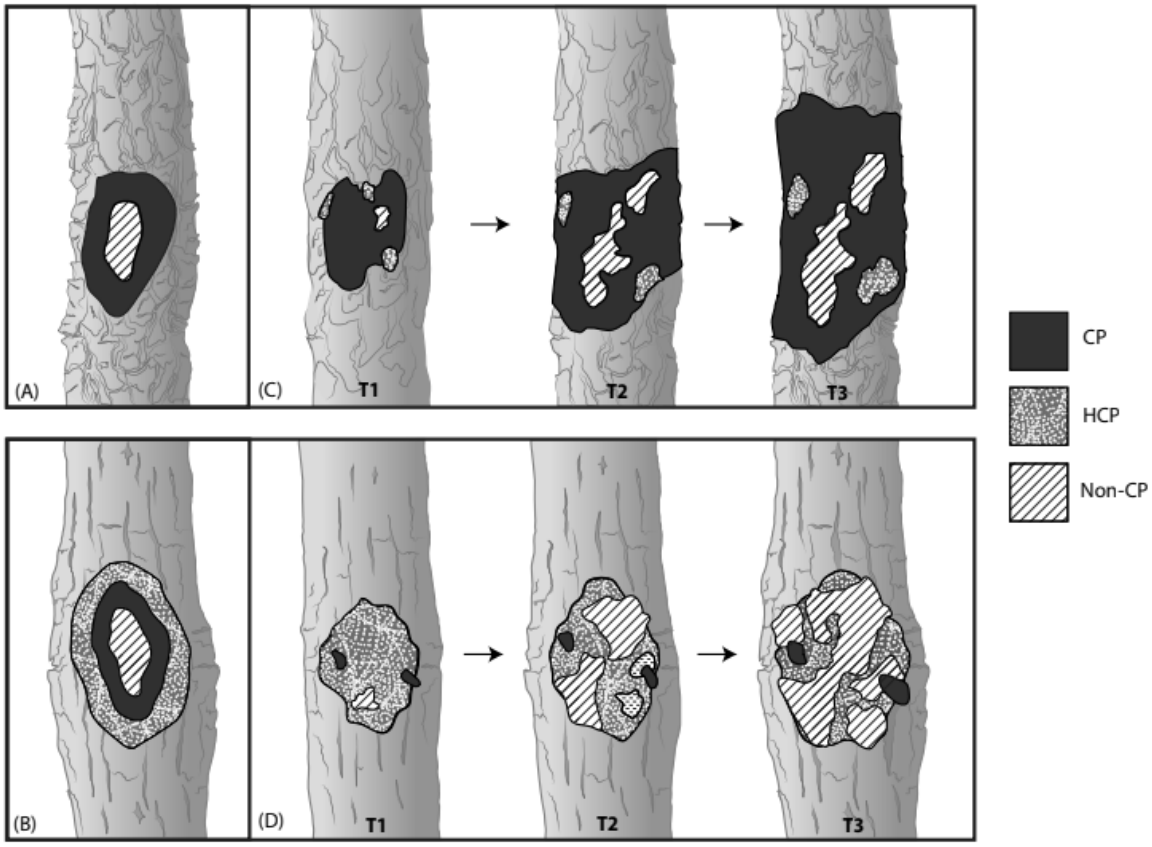


Figure 1: Original spatial model of fungal community within girdling cankers **(A)** and non-girdling **(B)** cankers on infected chestnut. **(C)** Updated time series model of mosaic fungal community in girdling cankers, which increase in virulent *C. parasitica* (CP) and total non-*C. parasitica* fungi (Non-CP) abundance relative to hypovirus-infected *C. parasitica* (HCP) and are likely to girdle in T2 or T3. **(D)** Time series of mosaic fungal community in non-girdling cankers, which increase in total Non-CP relative to CP; HCP decreases in abundance over time. Survivorship is expected to be higher in T2 and T3 compared to girdling cankers; however, survivorship over time is expected to decrease with the decline of HCP abundance in non-girdling canker

More surprising was the finding that the fungal community within a canker changes rapidly over time. Fungal communities were divided into four clusters based on the prevalence of CP, HCP and overall prevalence of Non-CP (Figure 2). Dynamics differed among population types (Table 1). New work is attempting to age cankers to determine if these temporal transitions are random or part of an orderly succession of cluster types.

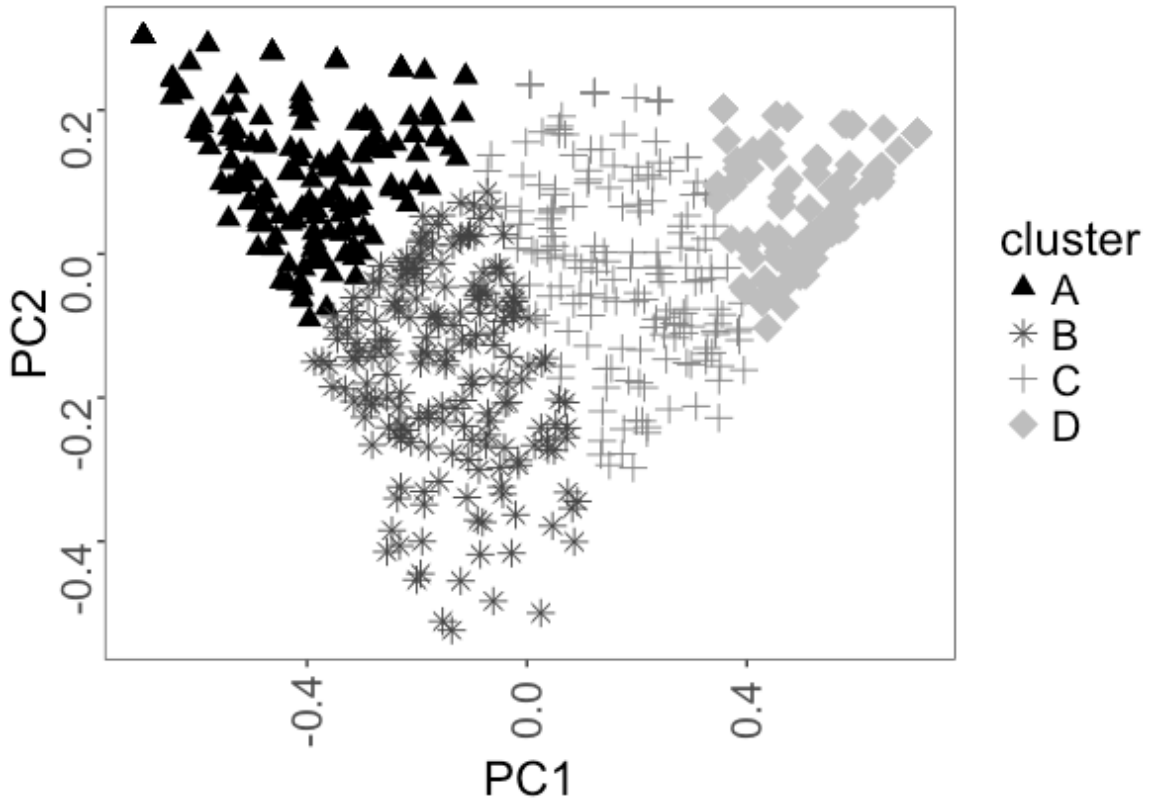


Figure 2: Principal Components Analysis (PCA) plot of four community clusters from canker-year observations (n = 633) of 61 fungal taxa sampled. PC1 separates observations based on the ratio of CP to HCP, where greater values represent greater amounts of CP relative to HCP in a canker. PC2 separates observations based on the amount of Non-CP, where smaller (negative values) represent more Non-CP in a canker relative to CP and HCP.

Table 1. Transition percentages among clusters over time. A. Summary data for recovering populations County Line, Roscommon and Frankfort; B. West Salem Wisconsin, and C. Epidemic populations Leelanau and Missaukee. Transitions are summed across all five years of the study.

A. Recovering populations.

	Cluster at time t + 1				
Cluster time t	A	B	C	D	Dead
A	38.3	38.3	12.8	5.3	5.3
B	27.4	48.4	18.9	1.1	4.2
C	18.2	51.5	21.2	4.5	4.9
D	6.3	28.1	31.3	9.4	25.0

B. West Salem.

	Cluster at time t + 1				
Cluster time t	A	B	C	D	Dead
A	9.1	22.7	40.9	13.6	13.6
B	33.3	11.1	11.1	18.5	25.9

C	12.1	24.2	27.3	18.2	18.2
D	7.9	18.4	18.4	15.8	39.5

C. Epidemic populations.

	Cluster at time t + 1				
Cluster time t	A	B	C	D	Dead
A	37.0	25.0	0.0	0.0	37.5
B	0.0	42.9	14.3	0.0	42.9
C	9.1	3.0	36.4	9.1	42.4
D	3.6	7.1	25.0	17.9	46.4

Angus Dawe, Mississippi State University

Current personnel:
Graduate students – Didi Ren (completed Ph.D. Aug 2018), Soum Kundu, Melanie Tran
Research Associate – Gisele Andrade

Identifying targets of the VIB-1 transcription factor, a component of the vic-mediated signaling pathway. Genetic regulation of vegetative incompatibility (*vic*) has been hypothesized to be a means of limiting the spread of hypoviruses, thus negatively impacting the utility of these agents as a mechanism for biological control. Compatible strains will form a stable heterokaryon, while incompatible strains will seal fused compartments that subsequently undergo programmed cell death. The transcriptional factor CPVIB-1 was found to be essential for the processes leading to vegetative incompatibility in *C. parasitica,* a model system for hypovirus-host interactions. In order to explore the direct targets of CPVIB-1, a detectable FLAG-tagged CPVIB-1 construct was transformed into the ΔCPVIB-1 strain. This protein was found to be able to substitute for the untagged (wild type) version both in the context of vegetative incompatibility and virulence (Fig 1; Fig 2). We were also able to identify that CPVIB-1 is post-translationally modified by the addition of ubiquitin, a common cellular component that targets proteins for degradation (Fig 3). It is not yet understood whether the modified or unmodified version of the protein is the biologically active form.

A ChIP-seq strategy was applied to reveal the precise binding motif and target genes potentially regulated by the CPVIB-1 protein. We found the GAGA-repeat motif was recognized and bound specifically by the CPVIB-1 protein (Fig 4). We found 264 genes that CPVIB-1 targeted with function in remarkably diverse biological processes, including cell fusion, transcription, translation, autophagy, telomere maintenance, response to oxidative stress, lipid and protein biosynthesis, and carbon metabolism (Fig 5).

From a former study, the TOR signaling pathway was demonstrated to be inactivated by both rapamycin and nutrient starvation, thus mimicking aspects of the *vic* response. Therefore, we have repeated the ChIP-Seq analysis using material treated with rapamycin prior to protein crosslinking. In this case, a slightly altered recognition sequence was returned (Fig 6), with potential targets numbering 357 in the genome (Fig 7). Interestingly, these represent almost an

entirely different set of genes identified without rapamycin stimulation, with only 21 being found in both sets (Fig 8).

Based on the known mode of action of rapamycin, we have developed a model for the activity of CPVIB-1 relative to the TOR signaling pathways. Usually, the functions controlled by the TOR2 pathway are rapamycin resistant compared to TOR1 because the FRB domain is hidden in TOR2 by the binding of STE20. In the absence of CPVIB-1, STE20 is reduced, thus exposing the FRB domain and rendering TOR2 also rapamycin sensitive. In conclusion, CPVIB-1 is a GAGA factor (GAF) interacting with a large collection of factors and genes that function in many different aspects of gene activity, chromosome structure and cell development and can be stimulated by rapamycin and nutrient starvation to inhibit the TOR signaling pathway.

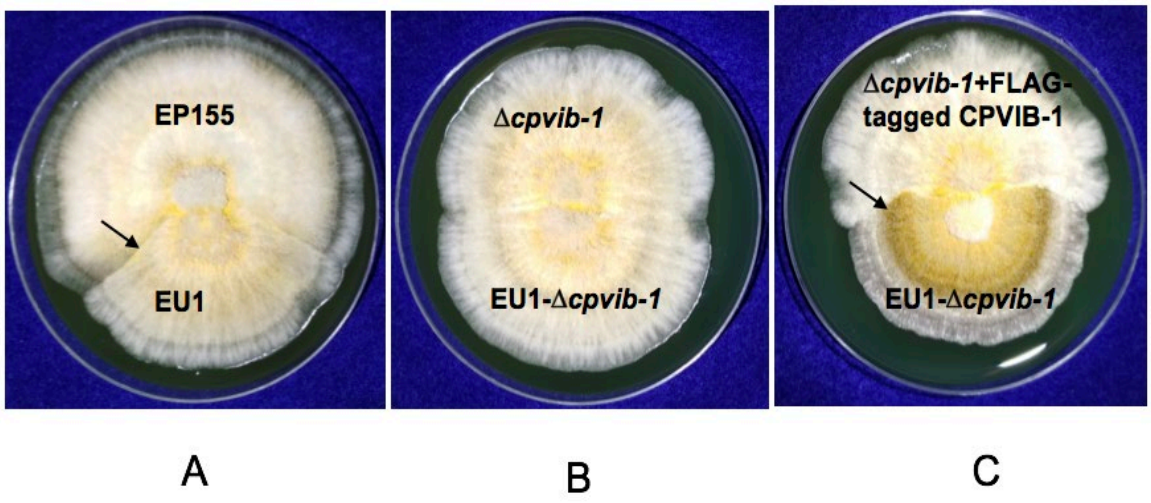


Fig. 1. Interactions between incompatible strains result in a barrage (A), in contrast to interactions when CPVIB-1 is absent (B). FLAG-tagged CPVIB-1 is able to restore barrage formation (C).

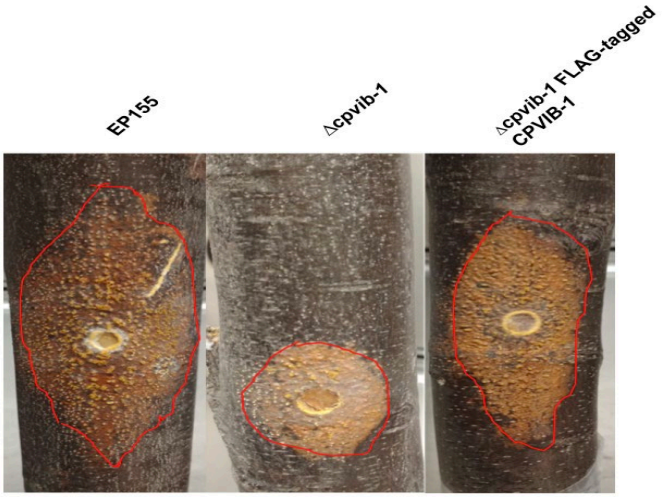


Fig. 2. The FLAG-tagged version of CPVIB-1 can restore the reduced virulence of the knockout strain.

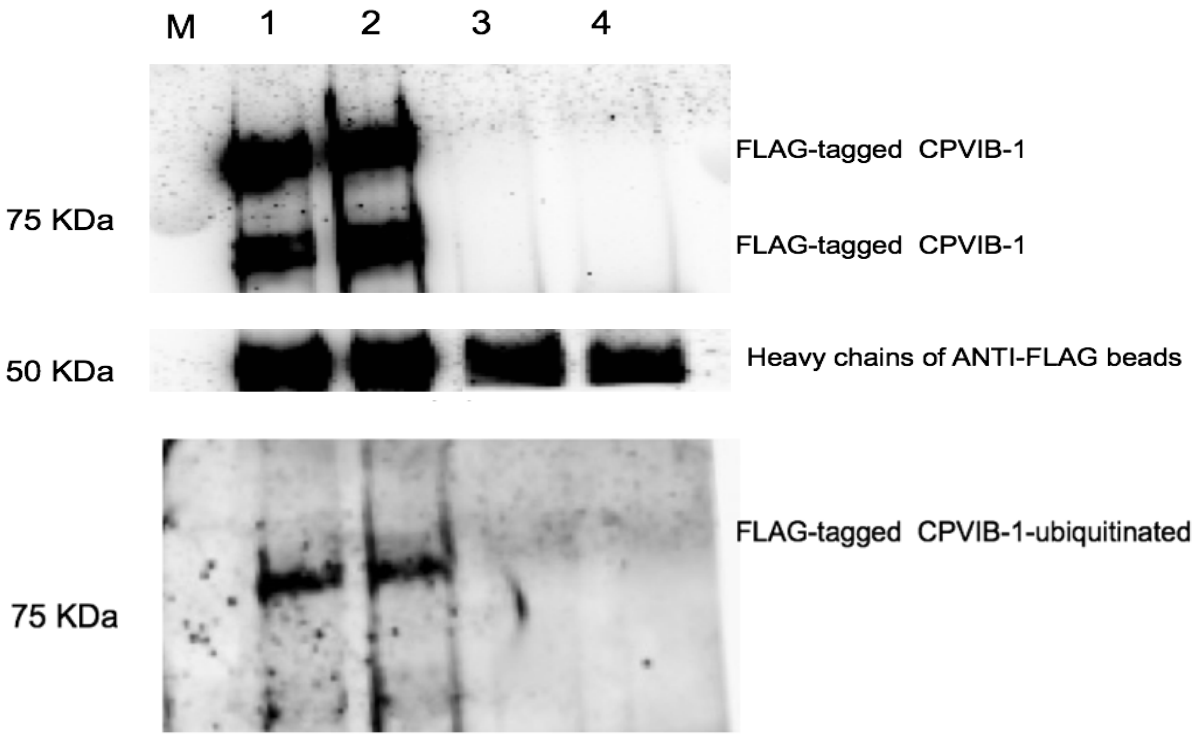


Fig. 3. Upper panel. Two bands are observed in immunoprecipitated protein using extracts from the FLAG-tagged-CPVIB-1 (lanes 1 and 2) compared to untransformed controls (lanes 3 and 4). Middle panel. Eluted heavy chains from the FLAG beads used as a loading control. Lower panel. Only the upper band is also detected by anti-ubiquitin.

Information for motif1

Reverse Opposite:

p-value:	1e-20
log p-value:	-4.760e+01
Information Content per bp:	1.530
Number of Target Sequences with motif	68.0
Percentage of Target Sequences with motif	24.73%

Fig. 4. The GAGA-repeat motif was the most recognized and bound by the CPVIB-1 protein.

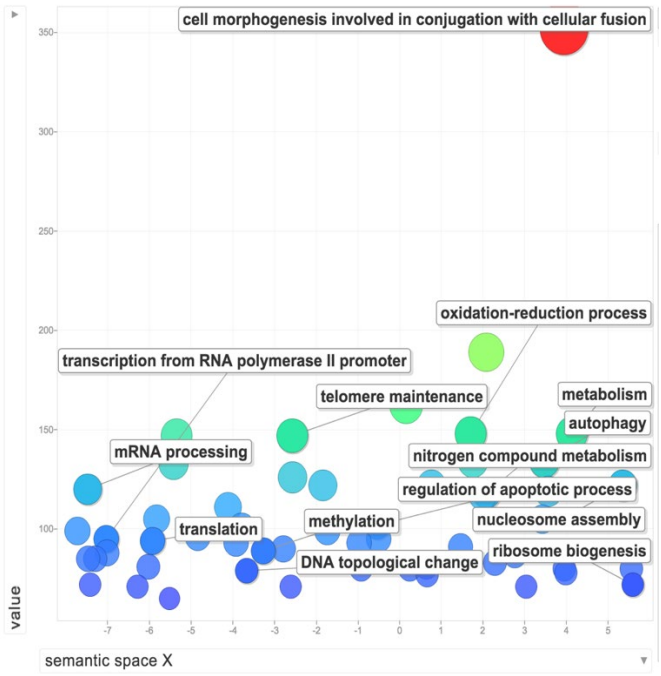


Fig. 5. CPVIB-1 targeted genes with predicted protein function in diverse biological processes.

Information for 1-CTTCTTYC (Motif 1)

Reverse Opposite:

p-value:	1e-7
log p-value:	-1.765e+01
Information Content per bp:	1.652
Number of Target Sequences with motif	116.0
Percentage of Target Sequences with motif	32.49%
Number of Background Sequences with motif	7826.0
Percentage of Background Sequences with motif	20.04%

Fig. 6. Fig4. The altered GAGA-repeat motif was the most recognized and bound by the CPVIB-1 protein in the presence of rapamycin.

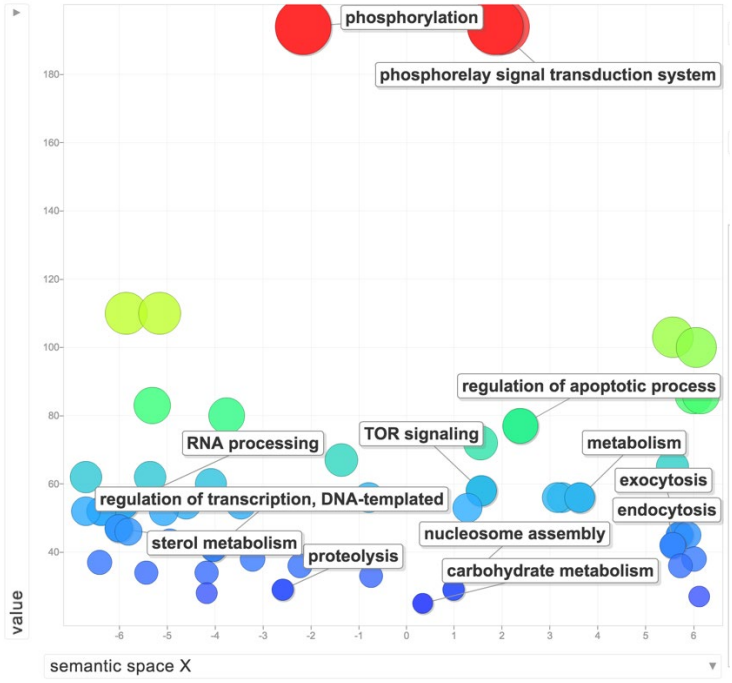


Fig. 7. CPVIB-1 targets regions of the genome encoding proteins predicted to have a wide variety of functions. The targets are altered by stimulation with rapamycin.

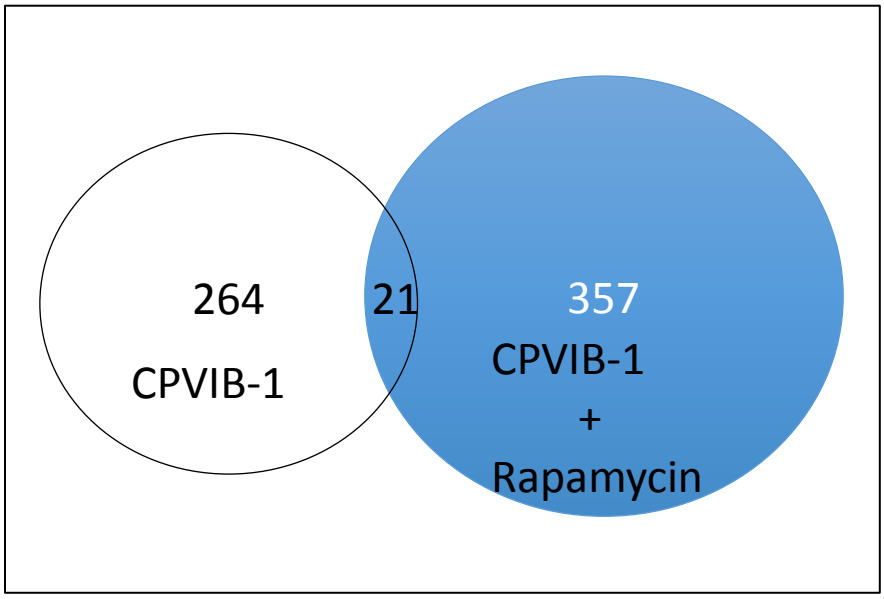


Fig. 8. Venn diagram of targets both with and without rapamycin stimulation.

Fred Hebard, The American Chestnut Foundation

Reasons behind the lack of widespread remission of blight in North America due to hypovirulence. Four reasons were given why hypoviruses have resulted in remission of blight in Europe but not in North America:

- Fewer vc groups
- More blight resistance
- Less competition from other tree species
- Differences in forest management

The definition and description of the terms, LSA (large surviving Americans) and SSA (small surviving Americans):

Definition. Large *Castanea dentata* greater than 32 cm (15 inches) in diameter at breast height that have been infected by *Cryphonectria parasitica* for at least 10 years are termed LSAs, smaller trees SSAs. LSAs and SSAs exist throughout the range of American chestnut.

Description. Cankers on LSAs and SSAs typically do not extend to the vascular cambium for many years, and are termed superficial. They can extend completely around and over 10 m up the trunk. The bark on such cankers is variously described as 'scruffy,' 'scurfy,' or 'cruddy.' When scruffy cankers are swollen, they have been described as 'big ugly.' In contrast, cankers in the process of killing *C. dentata* stems are not swollen, typically extend to the vascular cambium, have abundant stroma of *C. parasitica,* and are usually less than 1 m long.

The reasons for the existence of LSAs is Griffin *et al* (1983) concluded that survival of LSAs was due to a combination of genetic resistance and reduced virulence in most cases and one or the other in the rest. More recent studies indicate that reduced virulence could be due to numerous hyperparasites in addition to hypoviruses. Thus, LSAs and SSAs suggest that hypoviruses (and other hyperparasites) are widespread in North America: there is some disease remission occurring. This is in marked contrast to the general lack of disease remission on most *C. dentata*. We need to find out what is occurring.

Some basic assumptions:

- Absence of resistance in *C. dentata* is the null hypothesis.
- Survival of LSAs was associated with reduced virulence.
- Resistance was also detected in most LSAs studied by Griffin *et al* (1983).
- For LSAs without resistance, survival was most likely due to reduced virulence.

The frequency of LSAs and rarity of alleles for blight resistance:

- The alleles for blight resistance in American chestnut are expected to be rare due to the lack of selection prior to 1900 (Burnham, personal communication). LSAs occur at frequencies in the neighborhood of one in 100 million individuals of *C. dentata*. LSAs have increased in number over the last 40 years.
- Unlike LSAs, which are mostly single trees or in pairs, the trees in Michigan are in stands. This suggests that hypoviruses are important to recovery but not resistance, since so many trees are recovering. Rarity of random alleles for blight resistance also would argue for their rarity in founders of stand populations of *C. dentata* outside its native range, such as northwestern lower Michigan and Wisconsin.

Considerations for hypovirus deployment experiments:

- Chestnut blight is endemic on understory sprouts in mature forest but becomes epidemic on sprouts in clearcuts, leading to almost complete mortality 10 years after cutting (Hebard, 1982). Hypovirus deployment in clearcuts needs to begin 2-3 years after cutting before the disease becomes epidemic; otherwise inoculum of virulent strains becomes overwhelming. Remission in clearcuts may not be possible during the first epidemic, in parallel to the situation in Italy. There, blight started in one coppice cycle but remission associated with hypovirulence did not begin until the next coppice cycle (Mittempergher, 1978).

  Some experiments in addition to those described in the NE-temp1833 proposal include:

  - Ascospore concentrations in air should be sampled. No reports subsequent to 1915 have occurred in the US (Guerin *et al* 2001).
  - When progeny of SSAs from clearcuts were tested from resistance, no significant family differences were detected. SSAs in clearcuts may have survived longer when competing vegetation was removed.
  - The frequency of SSAs in clearcuts is about 1 per cut. Cuts in the Jefferson National Forest usually contain between 100 and 1000 sprout clumps of *C. dentata.*
  - Some isolates from SSAs in clearcuts were virulent but had reduced pathogenicity, including SG2-3.
  - Large samples of chestnut in clearcuts should be screened for resistance using strains with low pathogenicity such as SG2-3. Previous work with strains of average pathogenicity revealed no variation for resistance (Griffin *et al* 1983).

  A comparison of resistance in the forest to resistance after artificial inoculation is large (> 25 cm dbh) *C. mollissima* in China are more severely cankered than *C. henryi* or *C. sequinii* in forested situations, yet seedlings of *C. mollissima* test as more resistant after artificial inoculation. Progeny tests of the three species should be established in forest in their native habitat in China, such as at Dalaoling National Forest Park.

Resistance combined with hypovirulence may give good disease control as noted below:

- Anecdotally, Josh Springer's experience in Michigan is that blight can be controlled by treatment of cankers with hypoviruses on *Castanea* spp. and hybrids possessing intermediate levels of blight resistance but is difficult on *C. dentata* and unnecessary on *C. mollissima*.
- Gary Griffin at Virginia Tech has been saying this for years.
- TACF and WVU started an experiment designed to address this since 2006.

Paul Sisco, The American Chestnut Foundation (for Jared Westbrook, TACF and Jason Holliday, VA Tech)

Combining genomic with phenotypic selection in TACF's backcross breeding program and methods for incorporating transgenes to enhance blight resistance. To develop a population of genetically diverse, locally-adapted American chestnut trees resistant to chestnut blight, The American Chestnut Foundation (TACF) has been using a backcross breeding approach outlined by Burnham, Rutter, and French (1986) and refined by Hebard (2005). Blight-susceptible American chestnut trees from throughout its range were crossed to resistant

Asian chestnut species, primarily *Castanea mollissima*, and then backcrossed to different American chestnut trees three times, with selection for American type and intermediate levels of blight resistance in each generation. Beginning in 2002, trees from the third backcross generation with intermediate blight resistance were then intercrossed to increase the level of blight resistance, with a goal of developing a population of B_3F_2 American chestnut trees with high levels of blight resistance. This breeding strategy assumed a fairly simple genetic basis for blight resistance, 2 or 3 loci transferred from the Asian species that when homozygous would result in levels of resistance in American chestnut trees comparable to that of *C. mollissima*.

Several years of evaluation of B_3F_2 trees *per se* as well as progeny testing of their B_3F_3 offspring showed that the resistance captured so far is intermediate. Figure 1 illustrates the level of resistance of two populations of B_3F_2 trees, one coming from the 'Graves' B_1 tree and one coming from the 'Clapper' B_1 tree.

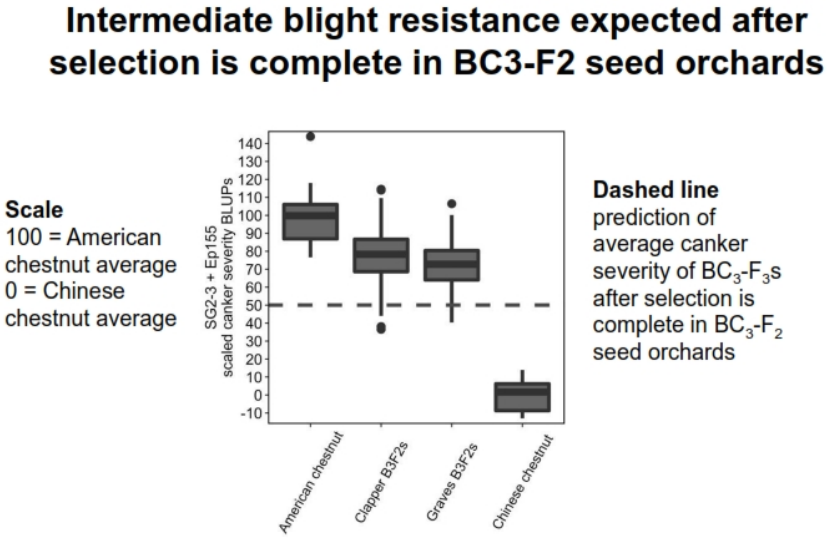


Fig. 1. Range of resistance expected in B_3F_2 trees after completion of selection

Selection so far has been on the basis of the blight-resistance of the B_3F_2 trees *per se* and of their B_3F_3 offspring based on several phenotypic characters: main stem alive/dead, canker size, sporulation of the fungus, amount of exposed wood, callus rating, and sunken vs. swollen cankers. But testing has shown there is a large environmental component to these traits that makes selection purely on phenotype inefficient. Westbrook (citation) has proposed development of an algorithm based on genome-wide sequencing that would, when combined with phenotypic scores, improve the accuracy of the selection process.

Additionally, TACF has added a program to breed for resistance to *Phytophthora cinnamomi*, which causes lethal root rot in American chestnut but not in the Asian chestnut species, which co-evolved with this pathogen. This pathogen is found in warm soils as far north as Pennsylvania. Fortuitously, the genetics of resistance to *P. cinnamomi* appears to be simpler than the genetics of resistance to chestnut blight, and some B_3F_2 trees in TACF's program are

resistant to this pathogen. Although phenotypic selection for resistance to *P. cinnamomi* has proved to be more efficient than phenotypic selection for resistance to chestnut blight, a genomic algorithm could also speed up the selection as well as allow for selection in the absence of this lethal, soil-borne pathogen (Fig. 2)

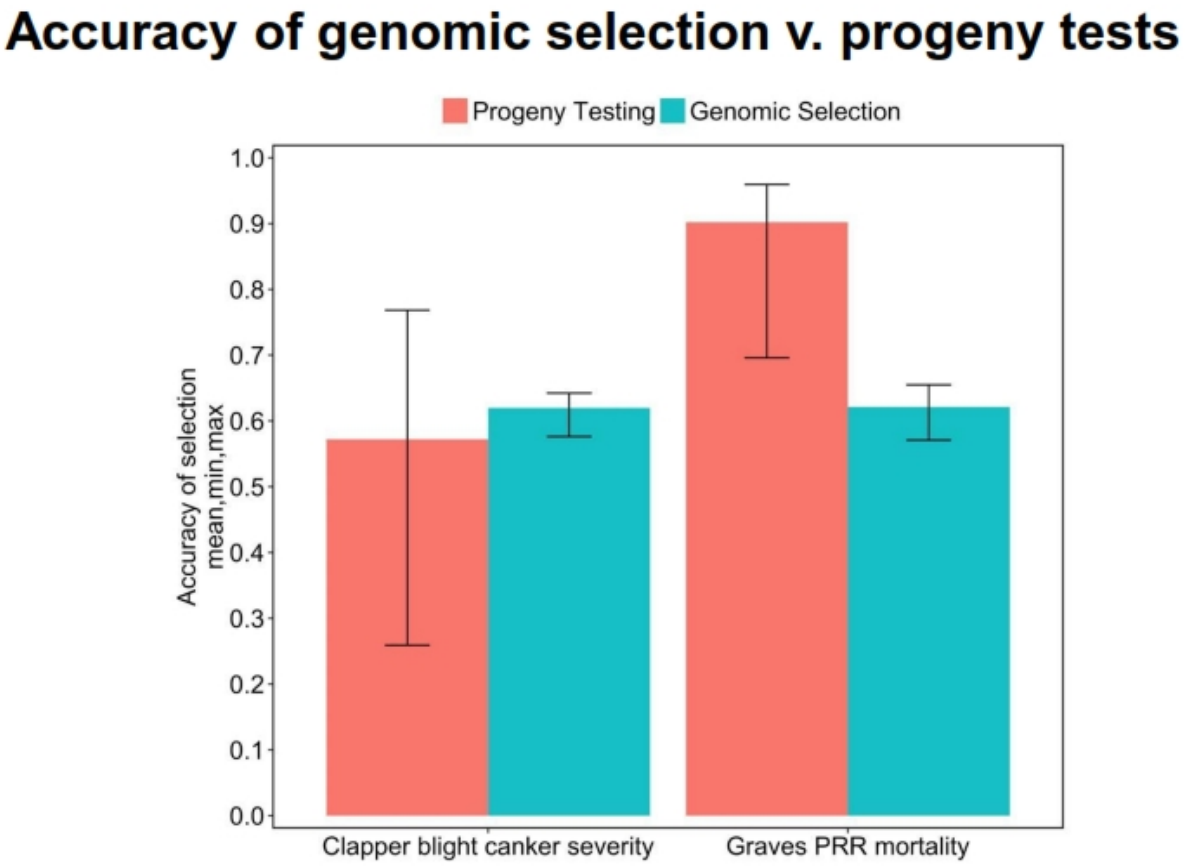


Fig. 2. Comparison of accuracy and variance of phenotypic vs. genotypic selection for resistance to chestnut blight (measured by canker severity) and to *P. cinnamomi* (measured by mortality after one-season exposure to the pathogen). Note the greater variance in phenotypic selection.

Finally, TACF is planting Germplasm Conservation Orchards (GCOs), reservoirs of American chestnut trees from throughout the range that could be used to cross to "Darling 58" or other blight-resistant transgenic trees being developed by Bill Powell's lab at SUNY-ESF. A broad genomics survey of American chestnut trees from Alabama to Maine now being undertaken will aid in selection of diverse and locally-adapted populations. Westbrook has proposed three generations of crossing to American trees, with techniques such as grafting and light manipulation to reduce the number of years necessary for each generation (Fig. 3).

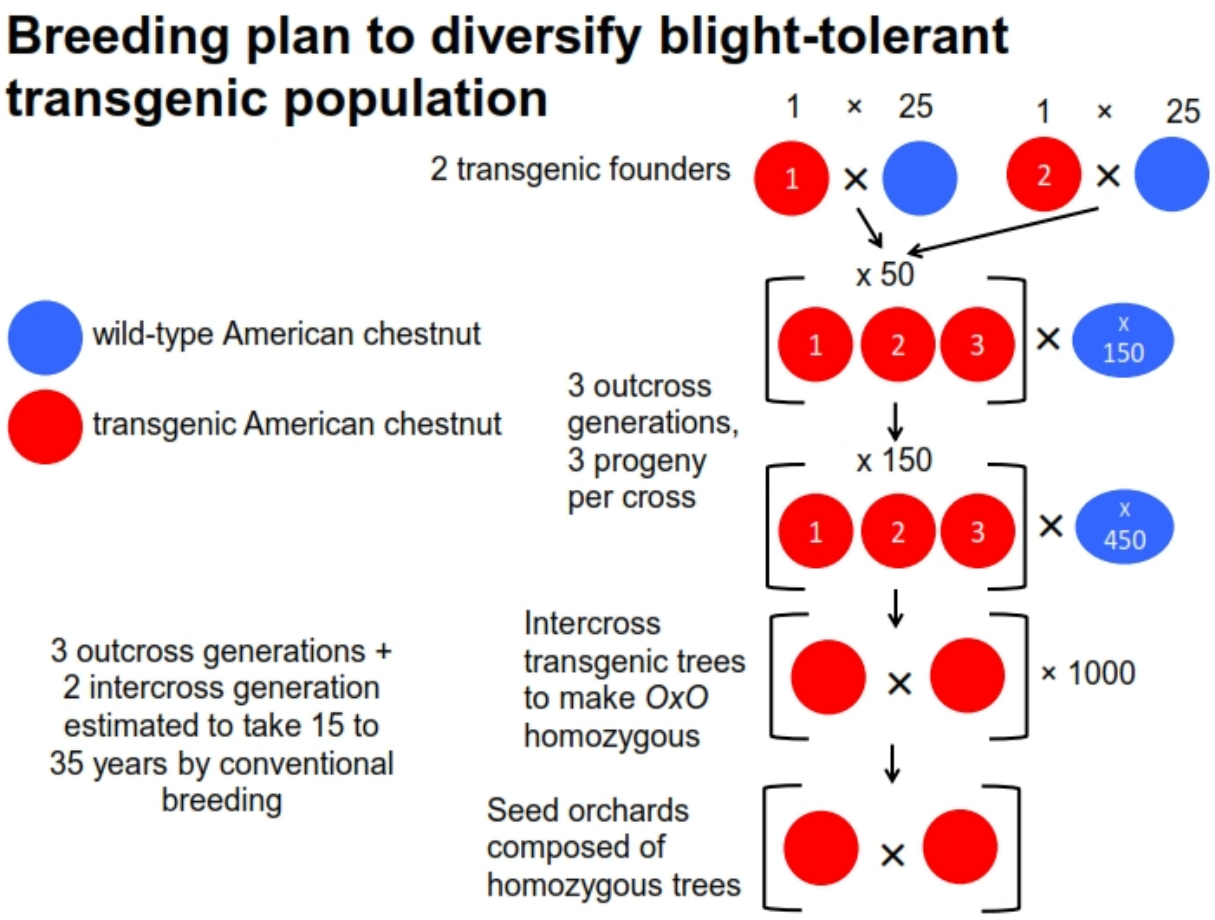


Fig. 3. Proposed crossing scheme to incorporate transgenes into a diverse and locally-adapted population of American chestnut trees.

<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

Andrew Jarosz, Dennis Fulbright (emeritus) and Matt Kolp, Michigan State University

Commercial chestnut orchards. Chestnut orchards in Michigan continue to be established. Dozens of new orchards have been planted in the last 3 to 4 years. The primary planting stock are European X Japanese hybrid cultivars including but not limited to 'Colossal', 'Bouche de Betizac', 'Precoce Migoule', 'Marigoule', 'Marsol', and 'Maraval'. A Japanese chestnut tree grafted as 'Labor Day' also is planted in some orchards. Very few large Chinese seedling orchards have been established in Michigan. The cooperative was established in 2002 and records indicate that the 2016 crop was the largest at about 212,000 pounds (just the cooperative). In 2017, a steep drop in yield occurred with only about 30,000 nuts produced (just cooperative). 2018 appears to be a good year for nut production in Michigan. A new, privately owned FACMA built chestnut harvester (Cimini 380) will be harvesting Michigan chestnut orchards in 2018. A new nut disease has been observed in Michigan. *Gnomoniopsis*

smithoglvyi has been observed on nuts in most Michigan orchards on nuts from both Chinese and European X Japanese trees. The parasitoid wasp of Asian chestnut gall wasp (ACGW) has been confirmed in Michigan orchards with ACGW. It appears to have arrived with ACGW.

Steve Jeffers, Clemson University (in collaboration with Chestnut Return Farm (Seneca, SC), The American Chestnut Foundation and USDA Forest Service)

Background Information: Phytophthora root rot on American chestnut. Our research focuses on Phytophthora root rot (PRR) of American chestnut and its hybrids, which is caused primarily by *Phytophthora cinnamomi*. While the story of chestnut blight (caused by *Cryphonectria parasitica*) and efforts to overcome this disease have been the subject of much public attention and numerous research efforts, much less consideration has been given to the role PRR has played in the demise of the American chestnut. Stems are killed back to the ground by chestnut blight, and these plants can re-sprout and survive. However, PRR is lethal; it destroys the root system and kills infected trees. Fortunately, *P. cinnamomi* is not present over the entire range of the American chestnut; it does not survive in northern climates where temperatures routinely get below freezing. Chinese chestnut, which was used as the source of resistance to *C. parasitica* in The American Chestnut Foundation (TACF) breeding program, also is resistant to *P. cinnamomi*, and genes for resistance to this oomycete pathogen are present in a proportion of hybrid seedlings that have been selected for resistance to *C. parasitica*.

P. cinnamomi is widely distributed in soils throughout the southern range of the American chestnut and has been killing American chestnut trees in southeastern forests since the early 1800s. The first report of PRR in the United States was in 1932, and the pathogen was tentatively identified as *P. cambivora*. This identification was in error, and the causal agent was correctly identified as *P. cinnamomi* in 1945. Until recently, *P. cinnamomi* was the only pathogen reported to cause PRR of American chestnut in the United States. However, in Europe, several species of *Phytophthora* are known to cause PRR of European chestnut—including *P. cinnamomi*, *P. cambivora*, and *P. cryptogea*—and other species have been found in soil associated with chestnut root systems. In 2017, S. R. Sharpe completed an MS Thesis in the Jeffers lab at Clemson University, and she reported isolating multiple species of *Phytophthora* from the roots of and soil associated with American, Chinese, and hybrid chestnut seedlings growing in field plots in several southeastern states. Based on her results, *P. cinnamomi* was isolated most frequently from seedlings and soil, but *P. cambivora* also was isolated from both seedlings and soil but much less frequently. *P. cryptogea* was isolated from several seedlings growing in a field plot in South Carolina; *P. heveae* was recovered from seedlings and soil at one site in North Carolina; and *P. quercetorum* only was recovered from one soil sample in Virginia. All four of the species isolated from seedlings were pathogenic to American chestnut seedlings in greenhouse trials.

Screening of Hybrid American Chestnut Seedlings for Resistance to *Phytophthora cinnamomi.* In collaboration with Dr. Joe James at Chestnut Return Farms in Seneca, SC and Dr. Paul Sisco with TACF, we have conducted an annual field trial to screen hybrid chestnut seeding continuously for 14 years: 2004-2017. During this time, we have developed a standard procedure for inoculating and evaluating chestnut seedlings using large tubs in an outdoor location. The two main objectives of this project were to determine if resistance was present in

hybrids selected for resistance to *C. parasitica* and then to identify sources of resistance for continued breeding efforts. During the current year, our 14 years of trials were summarized in a manuscript that was submitted to the journal *Plant Disease* in June 2018; currently, this manuscript is in the review process. One of the primary, outcomes of this research was that the hybrid cultivar 'Graves' was the best source of resistance to *P. cinnamomi*. Eventually, chestnut trees developed for the southern region will need to be resistant to both *P. cinnamomi* and *C. parasitica*.

In 2018, TACF moved the seedling screening project to the USDA Forest Service Resistance Screening Center (RSC) at the Bent Creek Experimental Forest in Asheville, NC. This decision was based on previous trials conducted at NCSU over several years and a preliminary trial at the RSC in 2017. The procedure at the RSC is a modification of the one used previously in the tub assay and involved planting seedlings individually in Deepots and growing these seedlings in a greenhouse. Plants were inoculated following the procedure previously used in the tub assay. Approximately 5 ml of V8-vermiculite inoculum was added to the top of each Deepot, inoculum was covered with sand, and plants were watered to prevent the inoculum from desiccating. After inoculation, seedlings were watered from the bottom through a unique recirculating irrigation system designed by the people at the RSC. Plants are being evaluated multiple times during the 2018 growing period with dead plants removed when found. Eventually, survivors will be evaluated for root rot severity using the 0 to 3 rating scale used in the tub assay: 0 = healthy, no lesions on roots; 1 = lesions only on feeder roots; 2 = severe root rot on the feeder roots or any lesion on the tap root; and 3 = 100% root rot. The advantage of screening seedlings at the RSC site is that more seedlings can be screened in less time. So far, the main disadvantage of the new system has been disease and pest problems due to the heavy plant density in the greenhouse—e.g., both powdery mildew and aphids have been issues.

Although seedlings grown outside in tubs is the standard method for screening seedlings for resistance, seedlings grown in a greenhouse in pots appears to be effective but further validation to compare results from the two assays are needed. The major drawback of any seedling assay is the time it takes to get results. The tub assay takes 8-9 months from planting seeds to evaluation, and the greenhouse assay takes 5-6 months from planting seeds to evaluation. Therefore, alternative in vitro methods that take less time to determine resistance of chestnut trees to *P. cinnamomi* would be helpful for breeding efforts.

An In Vitro Assay for Screening of Hybrid American Chestnut Trees for Resistance to *Phytophthora cinnamomi.* We are working to determine if it is possible to determine resistance of chestnut trees to *P. cinnamomi* using excised twigs in the laboratory. Mr. Andrew J. Gitto, a Research Assistant in the Jeffers lab, is working on this project. In this assay, we are using dormant, current-season shoots cur from trees in the orchard. Several shoots are cut from a single tree, and each shoot is then cut into twigs with uniform length. Twigs are randomized and then placed with the freshly cut proximal end pointing down in a disposable plastic culture tube that has inoculum in the bottom so that the cut end of the twig is in contact with the inoculum. Tubes are sealed and placed in an incubator for 7-14 days, depending on the temperature. We are working to optimize this assay and have experimented with incubation temperatures and times and various forms of inoculum. At the end of the incubation period, twigs are removed from tubes, the outer periderm is removed, and lesion lengths are measured. Lesions can be obvious or subtle depending on the type of chestnut inoculated. To

date, we have been able to consistently see a significant difference in lesion lengths on twigs collected from American and Chinese chestnut trees with lesion lengths on Chinese chestnut twigs shorter than those on American chestnut twigs. We also, have shown that twigs from selected hybrid chestnut trees at Chestnut Return Farms have significantly shorter lesion lengths that those on American chestnut twigs.

Detection and Recovery of *Phytophthora* species in Chestnut Soils. In collaboration with TACF, we continue to assay soils and symptomatic chestnut seedlings for presence of *Phytophthora* spp. – including locations where chestnuts are growing or might be planted. This is a service we have provided for 16 years (2003-2018). To date, we have isolated and stored several hundred isolates of *Phytophthora* spp. in a permanent culture collection. The procedure used is a simple baiting bioassay that was developed in our lab. In the past year, Sep 2017 – Aug 2018, we received samples from nine states: AL, GA, KY, MA, NC, PA, SC, TN, and VA. There were 16 submissions and 57 samples: 36 soil samples and 21 plant samples. *Phytophthora* spp. were detected in 26/57 samples = 45%. The species isolated primarily was *P. cinnamomi,* but we also isolated *P. cryptogea* from three seedlings in SC, *P. nicotianae* from one soil sample in TN, and *Phytophthora* sp. from one soil sample in AL. Eventually, we would like to determine the distribution of *Phytophthora* spp. in chestnut growing areas of the eastern United States.

Genetic Mapping the Resistance/Susceptible Response in Chestnut Seedlings to *Phytophthora cinnamomi* Infection. In collaboration with colleagues at University of Kentucky, USDA Forest Service, University of Tennessee at Chattanooga, and TACF—Dr. Tatyana Zhebentyayeva worked to identify QTLs for resistance to *P. cinnamomi* in several hybrid chestnut families based on the annual seedlings trials conducted at Chestnut Return Farms in SC. QTL analysis identified candidate genes on two linkage groups (LG): LG_E and LG_K. A manuscript describing this work is being prepared. Dr. Zhebentyayeva also initiated a study using transcriptome analysis and metabolome profiling to identify resistance loci in a hybrid chestnut family produced by a controlled cross. She reported on her projects in an independent presentation at this meeting.

Potential Future Research Projects. There are several projects the Jeffers Lab would like to initiate if funds can be secured to support the research. These are:

- Determine if chestnut trees resistant to *P. cinnamomi* also are resistant to other pathogenic species of *Phytophthora.* If not, these other species of *Phytophthora* must be included in future screening efforts to insure that seedlings resistant to all pathogenic species of *Phytophthora* are selected.
- Increase the diversity of the pathogen population when screening hybrid American chestnut genotypes for resistance to *P. cinnamomi* and to the other pathogenic species of *Phytophthora.* It will be important to include the most virulent isolates of each species in future screening efforts.
- Evaluate the efficacy of fungicides to protect American chestnut trees in conservation germplasm plantings and seed orchards from infection by *Phytophthora* spp. There are a number of oomycete-specific fungicides on the market that should be effective, but the efficacy of these products has not been tested on chestnuts.
- Evaluate the efficacy of fungicides to protect American chestnut trees in conservation germplasm plantings and seed orchards from infection by *Cryphonectria parasitica.*

Fungicide chemistry has changed quite a bit since fungicides were tested for efficacy against *C. parasitica*. Some of these newer fungicides may be effective at protecting chestnuts from the blight fungus.

Publications and Presentations

Peer-reviewed manuscripts:

- one submitted and in review – screening hybrid seedlings for resistance to *P. cinnamomi*
- one in preparation and almost ready to be submitted – QTL mapping of *P. cinnamomi* resistance loci

Andrew Gitto, Clemson University

Can excised twigs be used to identify resistance to *Phytophthora cinnamomi* in hybrid chestnut trees?

Current method for screening for resistance to *P. cinnamomi*:

Seedling inoculation trials:

- Clemson University
- North Carolina State University
- TACF
- USDA Forest Service Resistance Screening Center
- 14 years of annual trials

Seedling inoculation trials (current standard)

- Controlled or open pollination of mother trees
- Seed collection and stratification
- Seedling card and growth
- Seedling *P. cinnamomi* inoculation
- Seedling symptom scoring

Identifying tree resistance to *P. cinnamomi*, objectives include:

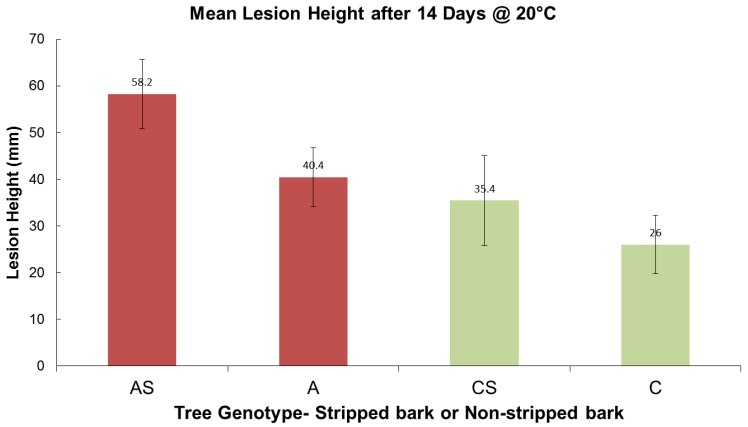
- Creating an additional test for resistance
- Complement seedling inoculation trial results to aid in screening

Using twig/shoot tissue to evaluate resistance

There are other plant assays that have used twig assays.

- Apple excised twigs were challenged with *P. cactorum* (1988).
- Australian eucalyptus trees (Jarrah and Marri) (1985)
- Banksia trees (1984).
- Hybrid chestnuts in Europe was assessed by Santos in 2014 comparing F_1 hybrid chestnut genotypes (*C. sativa x C. crenata* and *C. sativa x C. mollissima*). Most plantlets survived inoculations according to Santos.

Gitto used twigs with intact bark and stripped bark. Data from inoculations is as follows:



The time of twig collection may play a role in the growth of *P. cinnamomi*—actively growing tissue vs dormant tissue. Gitto is attempting to determine the optimal variable conditions to create the greatest difference in lesion heights between genotypes. This includes the type of isolate to use (highly vs moderately virulent), the incubation temperature and when to measure the lesions. Currently, Gitto cuts current season growth from mature trees that are 16" long and then cuts small twigs for replication. The twigs are dipped into inoculum and he's using American and Chinese chestnut as controls. His experience to date is that lesions on Chinese chestnut are much easier to define. For American chestnut, Gitto needs to strip the bark to better assess lesion length. In some American chestnut, the lesions are very diffuse and difficult to measure.

In summary:

- Preliminary results from the excised twig assay are positive.
- Assay still needs to be optimized.
- Validation requires testing trees from which seedling inoculation results are available – how many trees?
- Assay will be used to complement seedling inoculation results if successful.

Trent Deason, University of Tennessee, Chattanooga

Conservation and collection of *Castanea dentata* in the south. The current breeding program is limited to locating flowering trees. Trees infected with blight are in a cycle of growth, infection and die-back. Wild trees are relegated to the understory and they rarely receive sufficient light to produce flowers. Conservation and breeding efforts can be expanded by incorporating *ex situ* methods.

In biogeography:

- Repeated glacial events of the Pleistocene impacted the Eastern Hardwood Forest.
- Latitudinal cline in genetic diversity
    - Northern populations have fixed haplotype
- American chestnut compressed into one or few refugia locations in central Alabama and the Carolina's.
- Slow northward migration.
- The highest level of genetic diversity is in southern populations.

- Alabama is the center of diversity in that the highest frequency of rare alleles is located in that area.
- The high number of unique haplotypes exists in the Alabama, southern TN, northern GA region.

Ex situ conservation: graft propagation

- Southern populations, having greater genetic diversity and more frequent rare alleles, warrant targeted conservation.
- Urgency is needed to capture genes before trees succumb to pathogenic stress – control or reduction of pathogen pressure *ex situ* is possible.
- Graft-propagation allows clones to be grown in conditions suitable for flowering.
- Grafted plants can be planted out in germplasm conservation orchards (GCO) and maintained in the nursery.
    - GCOs allow for breeding and application of other technologies (GMO and biocontrol) to develop resistance.

The study area included

- Targeted scion collection from areas under-represented or not represented in TACF program
    - Southeast TN & Northwest GA
    - South-central TN & Northern AL
    - North-central TN & South-western KY
    - Western TN & Northern MS
- Identified sites were to visited twice – once in Fall of 2017 to confirm location and species, again in winter 2017-2018 for collection.

Scionwood from dormant trees were cut to the length of a standard gallon bag, rolled to remove excess air, and then doubled bagged to prevent desiccation. Bags were kept in an iced cooler for the duration of the collecting trip until they could be stored in a refrigerator (0°- 1°C). A variation of rootstock species and hybrids were selected to account for potential graft incompatibility. Resistance to pathogens obtained by non-*C. dentata* rootstocks was important as both *C. parasitica* and *P. cinnamomi* occur in the greenhouse and nursery. To prevent infection by either *C. parasitica* or *P. cinnamomi* rootstocks were treated with fungicide application.

Scionwood was collected form 13 sites (11 from a TACF announcement [6 from AL, 4 from TN and 1 from KY] and 2 from SERNEC (Southeast regional network of expertise and collections) herbarium database. Only one site (Cannon County, TN) was visited in the Fall 2017. The remaining sites were confirmed by a guide, GPS and winter identification.

Results of the scionwood collection are as follows:

- 3 of 4 Regions were collected from
- 33 individuals from 9 of the 13 sites identified
    - 6 AL sites within 5 counties:
        - Calhoun, Clay, Cleburne, Jefferson, Talladega
    - 3 TN sites in 3 counties:
        - Cannon, Hamilton, Henderson
        - Henderson not previously represented in TACF program

- 19 of 33 (57.6%) individuals are new sources of germplasm
    - Not previously captured in breeding program
    - Verified through TACF database local contacts

Grafting results are as follows:

- Of the 33 ortets, 13 out of 14 ramets survived to date.
    - 40 of 155 grafts (26%)
- Leaf samples were sent to TACF in support of range-wide landscape genomics study.
- One individual produced flowers 3 months after grafting.

Deason only visited one site during the growing season to confirm species identification. Expert guides and winter ID resulted in collection of "non-dentata" phenotypes. Five of 14 grafted individuals (36%) exhibited the phenotype of *Castanea pumila* var. *alabamensis*. It has American-like leaves and form but with a ciliated leaf margin.

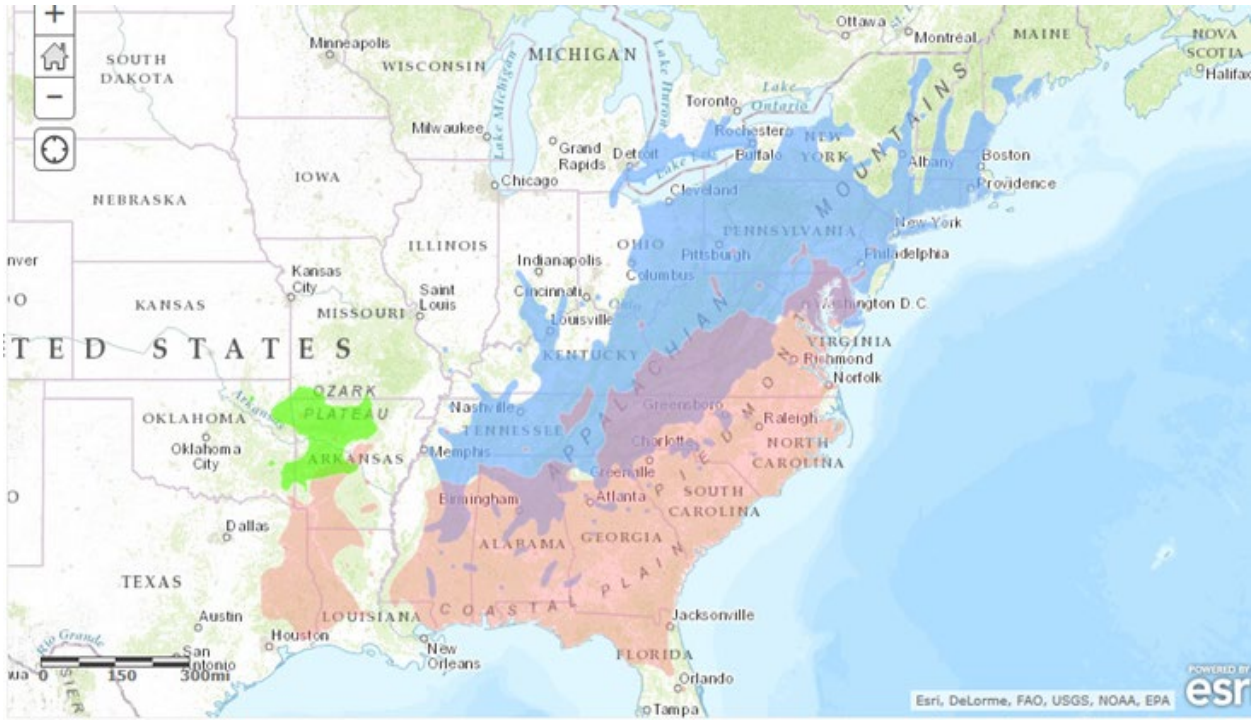
In conclusion:

- Southern populations of American chestnut harbor high genetic diversity and rare alleles.
- Conservation and propagation of rare genes is possible through grafting.
- Grafted plants can be grown in favorable conditions to allow flowering.
    - *Ex situ* conservation reduces pathogen pressure
    - Plants can be subjected to high light environments to accelerate flower production
- Pollen can be collected and used for breeding – broadening the genetic base of the TACF breeding program
- Container-grown grafted plants can be grown in high light environments in order to induce early flowering

Taylor Perkins, University of Tennessee, Chattanooga

Genome-wide SNP data and morphology uncover cryptic diversity in the chinquapins.

Castanea is a north temperate genus with several species. North American species include *C. dentata* (American), C*. pumila* var. *pumila* (Allegheny chinquapin) and C*. pumila* var. *ozarkensis* (Ozark chinquapin). The five Eurasian species are: *C. sativa, C. mollissima, C. seguinii, C. henryi* and *C. crenata*.



Distributions of North American *Castanea* species:
Blue shading = *C. dentata* (American chestnut)
Red = *C. pumila* var. *pumila* (Allegheny chinquapin)
Green = *C. pumila* var. *ozarkensis* (Ozark chinquapin)

American chestnut is a large canopy tree with three nuts/burr and glabrous leaves. Allegheny chinquapin is a variable taxon. It is a small shrub in the south but can grow to 10' tall further north. It was one nut/burr and it leaves have a wooly undersurface with stellate hairs. Ozark chinquapin is a medium-sized single stem tree with leaves that are larger than the Allegheny chinquapin.

Twenty-eight taxa or combinations have been proposed for chinquapins since 1753. American chestnut and Allegheny chinquapin often co-occur yet we can usually identify individuals to species. However, there are some populations (northern GA., SE TN and northern AL) where plants have a combination of traits from both species and we cannot identify these plants to species.

Perkins stated his problem:

- How much undiscovered biodiversity exists within the North American *Castanea*?
  - Unrecognized species?
  - Unrecognized varieties/subspecies?
  - Unique ecotypes?
  - Historical hybridization/gene flow between species?

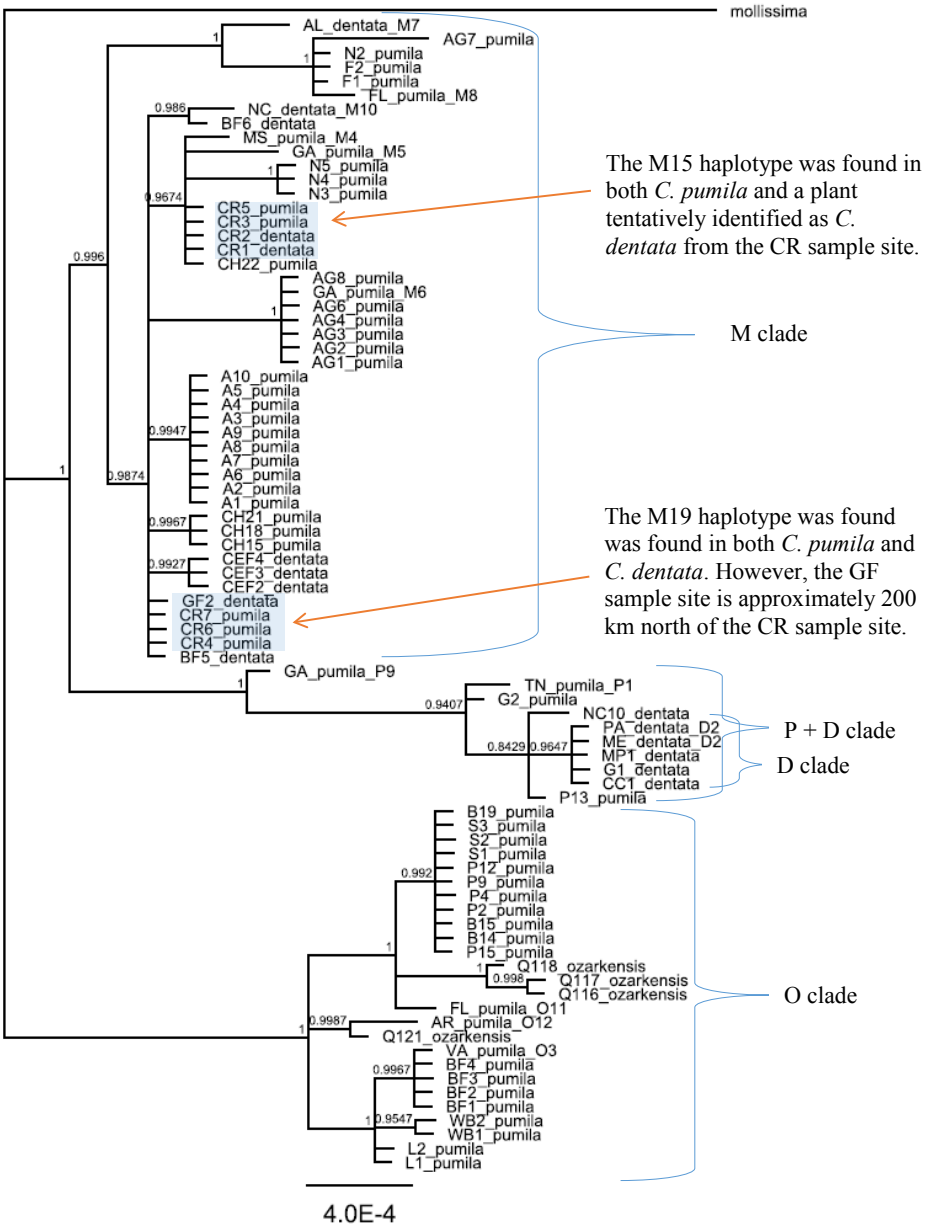
In 2016, Perkins visited 16 American chestnut sites (ME, PA, KY, TN, GA and AL); six Allegheny chinquapin sites (FL, GA, AL), two Ozark chinquapin sites and (AR and MO) and eight sites containing both American chestnut and Allegheny chinquapin (AL, GA, TN, NC). Perkins showed leaves of a mystery tree in Cleburne County, AL that had ciliated hairs on the leaf margin.

Perkins looked at 870 herbarium specimens from UNC-Chapel Hill. The samples were collected by W.W. Ashe in 1924-25 and the specimens Ashe identified as *Castanea alabamensis* match their AL specimen. Authors Camus (1929), Elias (1971) and Little (1979) identified these samples as a hybrid taxon, *Castanea x alabamensis*. A different opinion was offered by Johnson (1988) who identified the historical specimens as Ozark chinquapin.

Are the unusual chinquapin populations in northern AL explained by

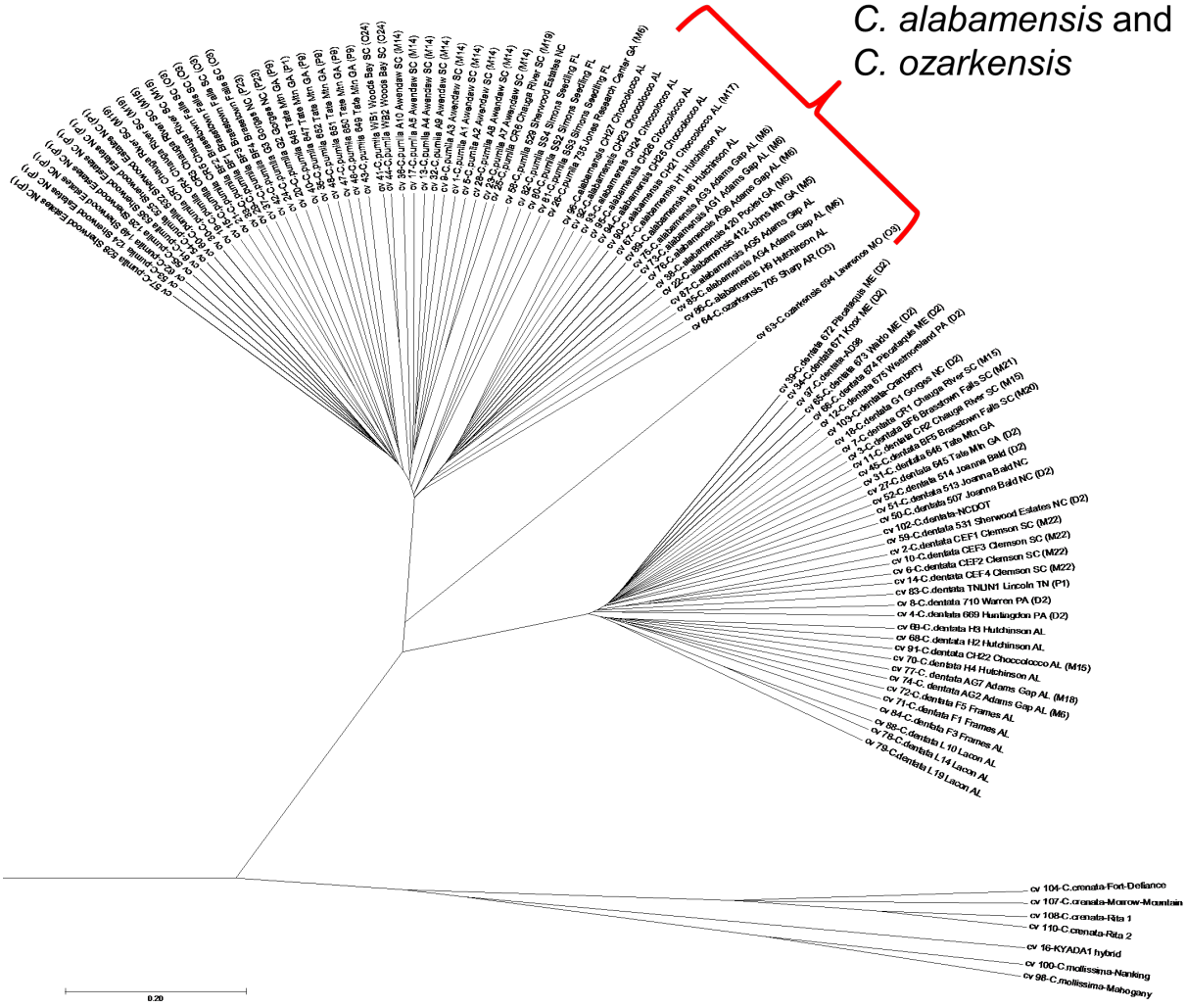
- Hybridization between *C. dentata* and *C. pumila*? (Camus, 1929; Elias 1971; Little 1979)
- Eastern disjunct populations of Ozark chinquapin? (Johnson 1988)
- A distinct species? Or variety?

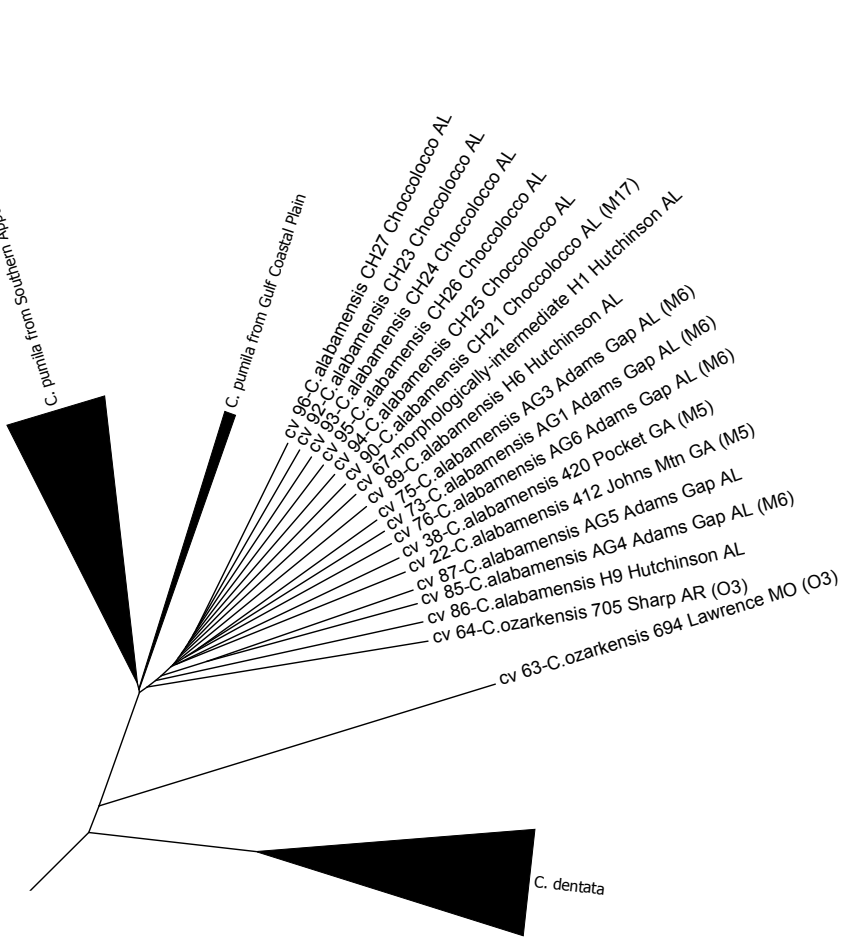
The following used sequence data from six noncoding chloroplast DNA regions of North American *Castanea* spp. plus *C. mollissima.*



Illumina sequencing and bioinformatics summary:

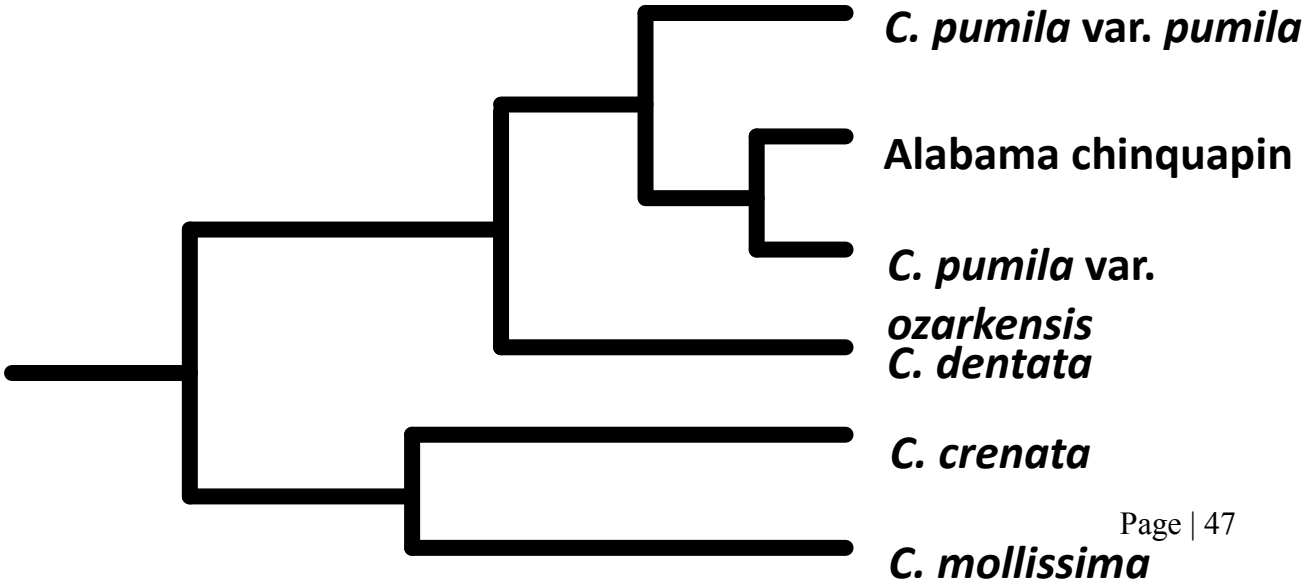
- Modified genotyping-by-sequencing (GBS) method of Elshire et al. (2011)
  - Double digestion of genomic DNA using *Pst1* + *Mse1*
  - Paired-end sequencing of 96 samples per lane using Illumina HiSeq 2500
- 385 million total reads obtained for 96 plants
- 378 million retained reads (97.74%)
- 2 of 96 samples failed sequencing
- 14 individuals < 1 million retained reads
- 4 million avg. retained reads per individual
- Alignment of Illumina reads to *C. mollissima* genome assembly V1.1 yielded 124,073 SNP loci for analyses





Identical chloroplast DNA haplotypes were sometime shared among C. denata and C. pumila. Structure analysis shows no evidence of American chestnut ancestry in Alabama chinquapin genomes. Also revealed by structure analysis is the extensive admixture among different botanical varieties of chinquapin.

Hypothesized evolutionary relationships of *Castanea* taxa inferred using GBS data is as follows:



Summary and future directions:

- Are the morphologically unusual chinquapin populations in Alabama explained by:
    - Hybridization between *C. dentata* and *C. pumila*?
    - An eastern disjunction of *C. ozarkensis*?
    - Poorly understood morphological variation within the *C. pumila* species?
- No evidence of *C. dentata* genomic contribution in Alabama chinquapins
- More sampling will be required to determine overall genetic similarity between Alabama and Ozark chinquapin populations, but Alabama chinquapins appear to be a distinct variety of *Castanea pumila.*
- Future work should investigate introgression vs. incomplete lineage sorting as causes of cytonuclear discordance

C. Dana Nelson, USDA Forest Service, Southern Research Station (SRS), Southern Institute of Forest Genetics (SIFG), Saucier, MS and Forest Health Research and Education Center (FHC), Lexington, KY

A research partnership between The University of Kentucky (UK), The Kentucky Division of Forestry, and the SRS has been formed in Lexington, the Forest Health Research and Education Center (FHC), that consists of collaborators primarily from SRS, UK, University of Tennessee (UT), Penn State, and Clemson. Bert Abbott serves as Biological Sciences Team Leader.

A free smart phone app, TreeSnap, has been developed through a National Science Foundation (NSF) project and in partnership with TACF that allows users to document American chestnut coordinates and provide supporting data and documentation such as photographs. In addition to American chestnut, TreeSnap has portals established with research partners working on ash, hemlock, American elm, Florida torreya and white oak. Meg Staton's Lab (University of Tennessee, UT) leads software development and Ellen Crocker (UK-FHC) leads education and outreach.

The Forest Health Initiative (FHI) project is ongoing for developing a reference genome sequence for Chinese chestnut genome in partnership with TACF, Penn State, UT and Clemson. (John Carlson (Penn State) will report). A project is ongoing for developing a reference genetic map for American chestnut in partnership with TACF and Virginia Tech (Jason Holliday (Virginia Tech) or Jared Westbrook (TACF) will report).

FHC projects in partnership with TACF and Clemson are providing Phytophthora root rot (P)RR) resistance and chestnut blight (CB) resistance genetic mapping.

a. Shenghua Fan (UK-FHC)-- Original analysis of CB resistance revealed that 3 genome regions (one each on LG_B, LG_F, and LG_G) controlled resistance in a 'Mahogany' based family. Current research with larger samples of progeny and markers in the same family supports the existence of resistance genes on LG_B and LG_G but implicates a different third region (LG_E). Ongoing analyses of 3 smaller backcross families including two additional Chinese resistance donors ('M16 ' and 'Nanking') also confirms the regions on LG_B and LG_E, plus an additional third region (LG_H). It appears that within a single backcross family, 3 regions control a significant amount of resistance, although each region plays more or less significant

roles depending on the resistance source. Three manuscript are in preparation (one to update the Chinese chestnut reference map and two on CB resistance mapping).

b. Determining the genes responsible within these regions is ongoing and being facilitated by previous genome mapping and sequencing efforts.

Other ongoing projects to be updated:

a. Application of FT-IR to predict resistance to PRR and CB in American-Chinese chestnut hybrids. Anna Conrad (FHC, now Ohio State) in collaboration with Jared Westbrook (TACF)

b. PCR-based detection of the PRR pathogen in environment samples of soil and water. Kenton Sena (UK-FHC), Tyler Dreaden (SRS-FHC) and Ellen Crocker (UK-FHC)

c. Surveying and DNA-based identification of native American chestnuts in Mississippi. Warren Nance (SIFG retired), Chuck Burdine (SIFG) and Randy Rousseau (Mississippi State)

d. American chestnut population genetics research with Oliver Gailing and students at Göttingen University in Germany. This includes a paper in preparation and one published in 2017 (Gailing, O. and C.D. Nelson. 2017. Genetic variation patterns of American chestnut populations at EST-SSRs. Botany 95:799-807).

Emily Dobry, Penn State-Erie, Behrend College

Isolation of *Gnomoniopsis smithogilvyi* from trees of *Castanea* exhibiting atypical cankers. A stand of 15 chestnut (5 American, 5 Chinese and 5 hybrids) were planted in North East, PA, and observed for signs of chestnut blight and overall success of the hybrids. In June 2018, several of the American and Chinese trees were observed to have cankers with atypical symptoms. Tree 4 had severe dieback and subsequently died, while two other trees showed signs of dieback. Samples were taken from near canker sights and isolated on PDA to determine whether *C. parasitica* was present. The 18 ITS regions were amplified with PCR followed by Sanger sequencing of the products and blast analysis. Two of the samples were determined to be *Gnomoniopsis smithogilvyi*, a pathogenic fungus that has only been recorded in the Eastern hemisphere and which induces symptoms resembling those of chestnut blight. It is unknown whether this fungus is ubiquitous or whether this may be a new invasive species affecting the health and survival of American chestnuts.

Tree	Fungal Species Identified	E-Value	Accession
American 2	*Epicoccum nigrum*	9E-98	MF281326.2
American 2	*Alternaria alternate*	2E-108	KX384640.1
Chinese 7	*Pestalotiopsis vismiae*	7E-130	EF055221.1
Chinese 7	*Phoma medicagnis*	2E-103	KF293988.1
American 4	*Gnomoniopsis smithogilvyi*	5E-152	KY6952232.1
American 3	*Gnomoniopsis smithogilvyi*	2E-125	KY695232.1

Business Meeting

Administrative Advisor, Bradley Hillman, thanked John Carlson for a great venue and a well-run meeting. Hillman noted that he has been a member of this group since 1986 and he is in a unique position as administrative advisor and also as a member of the project.

For the last 12 years, Hillman has been director for research for the NJ Agricultural Experiment Station, where he oversees federal funding. These multi-state projects work in that all those involved with state experiment stations get federal funding that states match 1:1. The federal funds or Hatch funds were begun in 1887. The purpose of the Hatch Act funding is to conduct agricultural research programs at State Agricultural Experiment Station in all 50 states. Directors at state experiment stations, must carve out ¼ of the Hatch funds to be spent on federally approved projects (i.e. multi-state research projects). A project is written; it then goes to an administrative advisor and then to all experiment station directors in the region for a vote. If approved at the experiment station director level, projects then must be approved at the federal level, USDA-NIFA. Multi-state projects are different than a coordinating committee (NECC). For coordinating committees, experiment stations directors are authorized to spend money on travel for participants, but nothing else (i.e. salaries). At the federal level, there are now fewer formula funds and more competitive funds. All experiment station directors deal with their funds differently. When this project was initiated, most of the initiators were associated with experiment stations. Increasingly now fewer people are associated with experiment stations. Since 1983, TACF has come of age and it has become a much stronger partner over time; however, TACF members to do not profit monetarily from a multi-state project.

We have just gone through a rewrite, so this is the last meeting of NE-1333. Hillman publically acknowledged the efforts of Fred Hebard who spearheaded the rewrite of the proposal for a new project. Since the multi-state process began, each time a new project is approved, a new number is associated with the new project. Thus, NE-1333 will be NE-1833 if approved. The reviews are back from the rewrite and Hebard has addressed the concerns of the reviewers. We are almost through with the whole process. Once the NE experiment station directors approve the new project it will move on to the USDA-NIFA level. Hopefully, the project will be approved by October 1, so money can be spent over the next five years.

Hebard pointed out and Hillman agreed that a multi-state project provides focus and structure and a meeting location where ideas can be shared.

Carlson then led a discussion regarding a new chair, vice-chair and secretary if the new project is approved. Matt Kasson (West Virginia University) agreed to be the chair for 2019 with a meeting to take place near Morgantown, WV. Andrew Jarosz (Michigan State University) is the chair-elect and Laura Barth (TACF, Meadowview) will take over for Mark Double as secretary.

Hillman stated that one of the unique members of the project is Mark Double. Hillman stated that Double has been a gift to the project for 36 years for his notetaking, and he publically acknowledged Double's efforts. Hillman has overseen 10 multi-state projects and no project has anyone who contributes efforts toward a project like Double. In recognition of his service, Hillman presented Double with a USDA-NIFA plaque.

A half-day field trip led by Sara Fitzsimmons, Steven Hoy and Kim Steiner followed the meeting. Members of the group were shown small-stem assays in the greenhouse, followed by a tour of plantings of B_2F_2 and B_3F_2 trees planted between 1997 and 2002.

Respectfully submitted, November 2018

Mark L. Double
West Virginia Univeristy

Publications:

Abbott, A. G., Zhebentyayeva, T., Sisco, P., Kubisiak, T., Olukolu, B., Jeffers, S. N., James, J., Hebard, F., Georgi, L., Staton, M. and Nelson, C. D. 2017. Genetic mapping of resistance to *Phytophthora cinnamomi* for American chestnut restoration in the southeastern United States. Abstract no. W340. Plant and Animal Genome (PAG) XXV Conference. San Diego, CA. 14-18 January 2017. Oral presentation and abstract published online: https://pag.confex.com/pag/xxv/webprogram/Paper24517.html

Cannon, N., Staton, M.E., Addo-Quaye, C., Islam-Faridi, N., Tomsho, L.P., Ficklin, S., Saski, C., Burhans, R., Drautz, D., Zembower, N., Schuster, S.C., Abbott, A.G., Nelson, C.D., Hebard, F.V. and Carlson, J.E. 2017, The Physical And Genetic Structure Of The Chinese Chestnut (*Castanea mollissima*) Genome, Plant and Animal Genome XXV Conference, San Diego, CA, 14-18 Jan 2017.

Carlson, J.E., Staton, M.E., Addo-Quaye, C. Cannon, N., Tomsho, L.P., Ficklin, S., Saski, C., Zhebentyayeva, T., Burhans, R., Drautz, D., Islam-Faridi, N., Wagner, T.K., Zembower, N., Schuster, S.C., Abbott, A.G., Westbrook, J., Georgi, L., Holliday, J., Nelson, C.D. and Hebard, F.V. 2017. The Chinese Chestnut Genome Project, IUFRO Genomics and Genetics of Fagaceae Conference, Shanghai Botanic Gardens, Shanghai, China, 1-3 Nov 2017.

Chang, S., Mahon, E.L., MacKay, H.A., Rottmann, W.H., Strauss, S.H., Pijut, P.M., Powell, W.A., Coffey, V., Lu, H., Mansfield, S.D. and Jones, T.J. 2018. Genetic engineering of trees: progress and new horizons. In Vitro Cellular and Developmental Biology-Plant. https://DOI.org/10.1007/s11627-018-9914-1

Conrad, A. O., Westbrook, J., Zhebentyayeva, T., Rodriguez-Saona, L., Bonello, P., James, J., Jeffers, S., Sisco, P., Hebard, F., Georgi, L., Staton, M., Audergon, J., Decroocq, V., Liu, Z., Dardick, C., Nelson, C. and Abbott, A. G. 2017. Metabolomics approaches for tracking biotic and abiotic stress performance in forest and fruit tree improvement programs. 34th Southern Forest Tree Improvement Conference (SFTIC). Melbourne, FL. 19-22 June 2017. Oral presentation and abstract published online: http://conference.ifas.ufl.edu/sftic2017/documents/SFTIC_2017_Program_Book.pdf

Double, M.L., Jarosz, A.M., Fulbright, D.F., Davelos Baines, A. and MacDonald, W.L. 2018. Evaluation of two decades of *Cryphonectria parasitica* hypovirus introduction in an American chestnut stand in Wisconsin. Phytopathology 108:702-710.

Elwood, E.C., Lichti, N.I., Fitzsimmons, S.F. and Dalgleish, H.J. 2018. Scatterhoarders drive long- and short-term population dynamics of a nut-producing tree, while pre-dispersal seed predators and herbivores have little effect. Journal of Ecology 106(3):1191-203.

Fitzsimmons, S.F., Collins, K.M., Westbrook, J., Saielli, T.M. and Brinckman, M.D. 2017. Germplasm conservation for species restoration: Examples from efforts to restore the American chestnut. In: Sniezko, Richard A., Man, Gary, Hipkins, Valerie, Woeste, Keith, Gwaze, David, Kliejunas, John T. and McTeague, Brianna A., tech. cords. "Gene conservation of tree species—banking on the future." Proceedings of a workshop. Gen. Tech. Rep. PNW-GTR-963. Portland, OR: US Department of Agriculture, Forest Service, Pacific Northwest Research Station. Vol. 963, p. 157.

Gailing, O. and Nelson, C.D. 2017. Genetic variation patterns of American chestnut populations at EST-SSRs. Botany 95:799-807.

Goldspiel, H.B., Newhouse, A.E., Powell, W.A. and Gibbs, J.P. 2018. Effects of Transgenic American chestnut leaf litter on growth and survival of wood frog larvae. Restoration Ecology, (In press).

Hein, K.M. 2018. Implementing Early Screening Methods to Detect Resistance to Phytophthora cinnamomi in Backcross Chinese-American Chestnut Hybrids. Honors thesis. University of Tennessee at Chattanooga, Department of Biological and Environmental Sciences. Chattanooga, TN. https://scholar.utc.edu/honors-theses/150

Jose, G., Gold, M.A. and Garrett, H.E. 2018. Temperate Agroforestry in the United States. Current Trends and Future Directions. In: Temperate Agroforestry Systems, 2nd Edition, Gordon, A.M., Newman, S.M. and Coleman, B. (eds.).

Kolp, M.R., Fulbright, D.W. and Jarosz, A.M. Inhibition of virulent and hypovirulent *Cryphonectria parasitica* growth in dual culture by fungi commonly isolated from chestnut blight cankers. Fungal Biology (in press) https:DOI.10.1016/j.funbio.2018.05.007.

Lovell, S.T., Dupraz, C., Gold, M.A., Jose, S., Revord, R., Stanek, E. and Wolz, K. 2017. Temperate Agroforesty Research—Considering Multifuncational Woody Polycultures and the Design of Long-Term Field Trials. Agroforestry Systems. https://DOI.org/10. 1007/s10457-017-0087-4.

Merkle, S. A., Gladfelter, H. and Jeffers, S. N. 2017. Development of an in vitro assay for resistance to *Phytophthora cinnamomi* in chestnut. 34th Southern Forest Tree Improvement Conference (SFTIC). Melbourne, FL. 19-22 June 2017. Poster presentation and abstract published online: http://conference.ifas.ufl.edu/sftic2017/documents/SFTIC_2017_Program_Book.pdf

Merkle, S.A., Vieitez, F.J., Corredoira, E. and Carlson, J.E. 2018. "*Castanea* spp. Chestnut." In Litz, R., Alfaro, F. and Hormaza, J. (Eds.), Biotechnology of Fruit and Nut Crops (2nd ed.). CAB International.

Mori, G.O., Gold, M.A. and Jose, S. 2018. Specialty crops in temperate agroforesty systems: Sustainable management, marketing and promotion for the Midwest region of the U.S.A. In: Integrating landscapes—Agroforesty for biodiversity conservation and food sovereignty. Montagnin, F. (ed.).

Newhouse, A.E., Oakes, A.D., Pilkey, H.C., Roden, H.E., Horton, T.R. and Powell, W.A. 2018. Transgenic American chestnuts do not inhibit germination of native seeds or colonization of mycorrhizal fungi. Frontiers in Plant Science, 9, 1046. https://DOI.org/10.3389/fpls.2018.01046

Steiner, K.C., Stein, B.S. and Finley, J.C. 2018. A test of the delayed oak dominance hypothesis at mid-rotation in developing upland stands. Forest Ecology and Management 408:1-8.

Steiner, K.C., Westbrook, J.W., Hebard, F.V., Georgi, L.L., Powell, W.A. and Fitzsimmons, S.F. 2017. Rescue of American chestnut with extraspecific genes following its destruction by a naturalized pathogen. New Forests, 48(2):317-336.

Westbrook J.W. 2018. Merging backcross breeding and transgenic blight resistance to accelerate restoration of the American chestnut. 2018. The American Chestnut Foundation's breeding and selection plan 2015 – 2025. www.acf.org

Westbrook J.W. and Jarrett B.T., 2017. Accelerated blight resistance screening with small stem assays. The Journal of the American Chestnut Foundation 31(1):22-24.

Westbrook J.W. 2017. Genomic selection for disease resistance in American chestnut backcross populations. The Journal of The American Chestnut Foundation 31(3):38-42.

Westbrook J.W. 2018. A plan to diversify a transgenic blight-tolerant American chestnut population. The Journal of the American Chestnut Foundation 32(3):31-36.

Westbrook, J., Hebard, F.V., Fitzsimmons, S.F. and Donahue, J. 2017. Status and future of breeding disease-resistant American chestnut. In: Sniezko, Richard A., Man, Gary, Hipkins, Valerie, Woeste, Keith, Gwaze, David, Kliejunas, John T., McTeague, Brianna A., tech. cords. "Gene conservation of tree species—banking on the future." Proceedings of a workshop. Gen. Tech. Rep. PNW-GTR-963. Portland, OR: US Department of Agriculture, Forest Service, Pacific Northwest Research Station. Vol. 963, p. 70.