Introducing disease resistance into American chestnut: state of the art

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The American Chestnut Foundation
S&T Spring 2020
Backcross breeding predicated on major effect genes for blight resistance

American and Chinese chestnuts are first crossed to help increase blight resistance.

F1 is the **first cross** to the American chestnut

B1 is the **first backcross** to the American chestnut

B2 is the **second backcross** to the American chestnut

B3 is the **third backcross** to the American chestnut

B3F2 is the **first intercross** to the American chestnut

B3F3 is the **second intercross** to the American chestnut

Breeding, testing and evaluation continues. TACF’s breeding program will continue to integrate additional sources of blight resistance into the breeding populations.

Three generations of backcrossing to recover American chestnut form

Charles Burnham

Two generations of intercrossing to enhance blight resistance
Backcross trees intercrossed to enhance blight resistance

\[ \text{BC}_3 \times \text{BC}_3 = \text{BC}_3\text{F}_2 \]

Resistance segregates among the progeny and currently approximately 1 out 150 \text{BC}_3\text{F}_2 selected
Selecting the 1% in chestnut seed orchards

“I plant trees and kill them”

- Selection nearly complete in 1 of 2 Meadowview seed orchards
- Approx. 500 trees remaining of 36,000 planted since 2002

Heavy lifting: Dan Mckinnon, Jim Tolton, Brandon Yanez
Questions

1. How do we measure blight resistance and what are the relative effects of genetics and environment on blight resistance?

2. Is blight resistance controlled by few genes or many genes and what are the implications for breeding?

3. What do we know about the mechanisms of blight resistance in chestnut and how can we learn more?

4. What does success look like?
What are the relative effect of genetics and environment on blight resistance?

Heritability - proportion of trait variability in a population that is due to genetic differences between individuals.

From King et al. (2010) Genome 53, 856-868
Measuring blight resistance at age <5: Artificial inoculation and canker severity assessment

- Backcross trees in seed orchards inoculated with weakly pathogenic strain of blight at age 2 – 3 years.
- Cankers rated 6 months to one year after inoculation
- 80% or more of trees with significant canker expansion removed
Resistance at age > 5 years: blight traits weakly heritable on individual backcross selection candidates

Main stem alive?  Cankers > 15 cm?  Cankers sunken?  Exposed wood?  Sporulation?

Phenotype = Age + Block + Genotype + Error

Genotype explains ~10% of the variation for most of these blight traits

<table>
<thead>
<tr>
<th>Trait</th>
<th>Clapper $h^2$</th>
<th>Graves $h^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main stem</td>
<td>0.08±0.04</td>
<td>0.06±0.06</td>
</tr>
<tr>
<td>Large cankers</td>
<td>0.25±0.09</td>
<td>0</td>
</tr>
<tr>
<td>Sunken cankers</td>
<td>0.09±0.05</td>
<td>0.07±0.05</td>
</tr>
<tr>
<td>Exposed wood</td>
<td>0.12±0.06</td>
<td>0.07±0.05</td>
</tr>
<tr>
<td>Sporulation</td>
<td>0.07±0.06</td>
<td>0.07±0.05</td>
</tr>
</tbody>
</table>

Parent condition index = sum of blight trait probabilities
Assessing genetic component of blight resistance with progeny tests

Unselected BC$_3$F$_2$ seed orchard

Partially selected seed orchard

Artificially inoculate & cull susceptible

Open-pollination among BC$_3$F$_2$ survivors

Select parent whose progeny have smallest cankers

Artificially inoculate BC$_3$F$_3$ progeny from each selection candidate
Average canker severity of progeny of selection candidates moderately heritable

Canker severity = canker rating + canker length 6 mo. post-inoc

- Clapper, 346 families, $h^2_{\text{family}} = 0.67 \pm 0.06$
- Graves, 197 families, $h^2_{\text{family}} = 0.59 \pm 0.09$
Small stem assays accelerate progeny testing for blight resistance

Inoculate first year seedlings with highly virulent strain of chestnut blight fungus (Ep155)

Measure
- Days to wilt
- Survival proportion within families 18 weeks post-inoculation
Genomic selection increases speed and accuracy of selection in seed orchards

> 3,000 BC$_3$F$_2$ selection candidates genotyped for genomic selection

> 800 BC$_3$F$_3$ families progeny tested in orchards or in SSAs

> 2000 BC$_3$F$_2$ selection candidates phenotyped for long-term blight resistance

DNA marker

Gene involved in blight resistance

Genotyping by Qian Zhang and Jason Holliday, Virginia Tech
Genomic prediction enables estimation of individual tree resistance and progeny resistance for all selection candidates

Different subsets of selection candidates phenotyped, progeny tested and genotyped
How genomic prediction works

Full sibs expected to share 50% of genome

A better weighted average

DNA markers trace variation around expected pedigree-relationships

Pedigrees weight sibs equally

Genomics + pedigree weights sibs in proportion to true relatedness
How genomic prediction works

Resistance of non-phenotyped individuals predicted from average of resistance of relatives weighted by strength of relationship

Genomic data estimates genome sharing between relatives more accurately than pedigree
Genomic selections made based on sum of parent and progeny resistance

**Blight selection index =**

\[
\text{Parent Condition Index} + \frac{3}{4}(\text{progeny blight resistance}) + \frac{1}{4}(\text{ssa survival})
\]

Correlations with SSA resistance weaker

\[r = 0.34\]

\[r = 0.23\]
Virtual tour of seed orchard selections

American chestnut

D4-27-78
93% American
2003

D2-18-122
91% American
2006

Photos: Eric Jenkins
Virtual tour of seed orchard selections

W6-8-121
83% American
2009
edge effect?

D9-18-1
77% American
2008

D8-7-9
67% American
2011

Photos: Eric Jenkins
Virtual tour of seed orchard selections

D1-7-69
68% American
2010

D3-17-73
43% American
2006

D1-17-99
42% American
2006

Photos: Eric Jenkins
Is blight resistance controlled by few genes or many genes?

**Few genes:**
High levels of resistance possible with high *C. dentata* ancestry

**Many genes:**
Strong tradeoff between resistance and *C. dentata* ancestry
Tradeoff between blight resistance and American chestnut ancestry implies resistance is controlled by many genes.

BC₃F₂ (expected to be 94% American chestnut) vary from 35% to 100% American.
Mapping regions of hybrid genomes associated with blight resistance

Infer regions of Chinese and American chestnut ancestry in hybrid genomes

Scan the hybrid genomes for regions that are correlated with blight resistance

Figure from Mackey, T. F. Quantitative trait loci in Drosophila. Nature Reviews Genetics 2, 13 (2001)

Analytical pipeline
John Lovell
HudsonAlpha
Regions on all 12 chromosomes associated with blight resistance

Total length of QTL = 110 Mb (~1/7th of genome length)

Avg QTL interval = 6 Mb

Min QTL interval = 0.7 Mb

Avg. trait variance explained by single QTLs = 3%

Total trait variance explained by all QTL = 2% to 57% depending on trait & population

Variance explained by largest single QTL = 13%

Clapper and Graves have unique and shared QTLs
Subset of genomic regions associated with blight phenotypes of BC$_3$F$_2$ parents also associated with average canker severity of BC$_3$F$_3$ progeny

- QTLs explain 15.3% and 17.5% of the variation in BC$_3$F$_3$ progeny canker severity in ‘Clapper’ and ‘Graves’ populations, respectively.
- Likely additional QTLs, but population of BC$_3$F$_2$ mothers whose progeny have been screened for blight resistance too small to detect additional QTL.
No evidence that crossing **Clapper** and **Graves** enhances resistance of BC₃F₂s

Infer paternity of BC₃F₂ with genotypes of BC₃F₂s and candidate BC₃ fathers

Candidate **Clapper** and **Graves** BC₃ dads

Known ‘Clapper’ BC₃ mom

Inferred dad

Genotyped BC₃F₂ progeny

Paternity analysis by Fred Hebard
Breeding: The path forward

• Increase resistance by increasing Chinese chestnut ancestry
  • As much as necessary as little as possible
• Cross inferior backcross lines with OxO
• Moderate expectations
Finding a balance between blight resistance and American traits in traditional breeding

Increase stringency of selection at BC_3/BC_4

Increase blight resistance by intercrossing BC_3F_2s with F_1s and BC_1s

Breeding, testing and evaluation continues. TACF’s breeding program will continue to integrate additional sources of blight resistance into the breeding populations.
Plant male sterile $F_1$'s in $BC_3F_2$ seed orchards to generate “Better BC1's” with enhanced resistance.

Figure and photos by Paul Sisco
Increase stringency of selection for blight resistance in chapter backcross populations

**Genotype** to infer % American ancestry in chapter backcross trees

**Phenotype** trees inoculated > 2 years ago for traits indicative of blight resistance/susceptibility

251 of 521 trees candidate backcross selections genotyped to date have >95% American chestnut ancestry and are likely to have inferior blight resistance

Trees with inferior blight resistance may be crossed with F₁s to create BC₁s or bred with transgenic trees
Outcrossing transgenic blight-tolerant American chestnut an efficient method to “rescue” genetic diversity

Apply transgenic-OxO pollen to wild-type trees

50% of progeny expected to inherit OxO
Outcross OxO to wild-type trees over five generations to trees to increase diversity and minimize inbreeding

Increasing numbers of WT parents each generation is an efficient method to increase $N_e$ and minimize inbreeding (see Westbrook et al., 2019 for details)
# Results of OxO outcrosses in 2019

Individual nuts given a unique ID

Core from individual nuts tested for OxO

Colorimetric assay indicates inheritance of OxO

<table>
<thead>
<tr>
<th>Location</th>
<th>N mother trees</th>
<th>N seeds</th>
<th>N (%) OxO positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUNY</td>
<td>20 WT,</td>
<td>893</td>
<td>337* (44%)</td>
</tr>
<tr>
<td>Meadowview</td>
<td>3 LSA, 2 BC$_3$F$_2$</td>
<td>519</td>
<td>219 (42%)</td>
</tr>
<tr>
<td>Purdue</td>
<td>10 WT</td>
<td>638</td>
<td>303 (44%)</td>
</tr>
</tbody>
</table>

* 795 nuts tested for OxO to date

Photos: Brandon Yanez
What do we know about the biology of the blight infection and resistance?

*Cryphonectria parasitica* mycelial fans grow into the bark, cause necrosis, and girdle the stem.

Photo: Fred Hebard
**OxO protects plant from oxalic acid (OA)**

OA leaf disk assay (no fungus)

- Green – living tissues
- Brown – dead tissues

Lower bar = more tolerant

- Oxalate-CoA ligase
- Oxalyl-CoA decarboxylase
- Formyl-CoA hydrolase
- Formate dehydrogenase

**Chinese chestnut has no Oxalate oxidase, so...**

**OA tolerance trait**

(Dakota Matthew’s assays)
Mycelial fans form more rapidly in American chestnut relative to Chinese chestnut, which is resistant to blight.

Photo: Fred Hebard
The rate and thickness of cell wall lignification and wound periderm formation in bark surrounding blight infection is correlated with resistance.

Chinese chestnut forms thicker periderm faster than American chestnut.

Figure: Fred Hebard
Strategy for discovering additional candidate genes for blight resistance

Step 1: Assemble chestnut reference genomes

Chinese chestnut (source of resistance)  American chestnut

Step 2: Scan hybrid genomes for regions correlated with blight tolerance

Position along chromosome

Correlation of DNA variants with canker severity

Location of blight resistance gene

Step 3: Compare gene expression in Chinese chestnut and American chestnut stems after blight infection
Proposed experimental design for chestnut blight transcriptome/metabolite timecourse

Clonally propagate 6 genotypes via grafting
  2 Chinese chestnut
  2 F1s
  2 American chestnut

Inoculate stems with C. parasitica

Harvest stem tissue for analysis at 3 timepoints
  Before inoculation
  1 – 7 days after inoculation
  7 to 14 days after inoculation

Contrasts
  American v. Chinese
  Two Chinese and F1s
  Allele specific expression in F1s
  Large surviving v. susceptible American chestnut

Total samples = 54
Chinese chestnut cisgenes enhance blight resistance in American chestnut

Transgenic American chestnuts with candidate genes from Chinese chestnut
Cisgenic American chestnuts in the field

American chestnut

Cisgenic American chestnut with CBS1 gene

Chinese chestnut

Photos: Sara Klopf
CRISPR applications: Adjacent insertion of multiple resistance genes to simplify inheritance of polygenic resistance.
CRISPR applications: Insertion of resistance gene(s) into the same genomic location in multiple founders

- Alleviate founder bottleneck when resistance gene(s) are bred into wild populations
- Insertion into same genomic location minimizes probability of gene silencing when different founder lines intercross
## What does success look like?

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Breeding</th>
<th>Biotech</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main stems survive indefinitely with blight infection.</td>
<td>Yes if Chinese ancestry increased</td>
<td>Likely but too early to assess durable resistance</td>
</tr>
<tr>
<td>Trees grow at rates and maximum heights more similar to American chestnut than Chinese chestnut.</td>
<td>Maybe not if Chinese ancestry increased</td>
<td>Yes</td>
</tr>
<tr>
<td>Leaf, twig, and nut characteristics similar if not indistinguishable from American chestnut</td>
<td>Maybe not if Chinese ancestry increased</td>
<td>Yes</td>
</tr>
<tr>
<td>Populations adequately represent the diversity and adaptive capacity remaining in <em>C. dentata</em></td>
<td>Maybe, but need to compare diversity in BC and wild populations</td>
<td>Yes if outcrossed to wild populations</td>
</tr>
<tr>
<td>A subset of the population has tolerance to both <em>Cryphonectria parasitica</em> and <em>Phytophthora cinnamomi</em>.</td>
<td>A subset of BC trees has PRR resistance.</td>
<td>Breed with PRR resistant BC trees to combine resistance.</td>
</tr>
</tbody>
</table>
Additional breeding to combine blight and PRR resistance

PRR resistant BC3-F2 selections

Blight resistant transgenic outcross progeny

Intercross to increase PRR resistance

X

Select for PRR resistance and deploy

2 generations of breeding and selection
Possible to generate hybrids with > 80% American chestnut ancestry AND high resistance to *P. cinnamomi*

Most PRR-resistant selections have >80% *C. dentata* ancestry.
Fewer, larger effect QTL associated with resistance to *Phytophthora cinnamomi*

Two QTLs on Chromosome 5 explain a total of 20% of variation in PRR mortality.

American chestnut backcross trees that survived inoculation have partial resistance to *P. cinnamomi*
Germplasm conservation for diversifying transgenic trees

Objective: Collect seed or graft 1000 wild-type American for use in breeding with blight-tolerant transgenic trees

Document locations of wild trees with TreeSnap

Mark tree for winter scion collection

Graft wild trees that do not flower in forest

Collect seed from rare flowering trees
3BUR: Breeding, Biotechnology, and Biocontrol United for Restoration

Conservation of backcross lines

Selection for blight tolerance

Backcross breeding

Selection for Phytophthora resistance

Genomics

Selection for Phytophthora resistance

Marker-assisted introgression

Germplasm conservation

Discovering genes for resistance

Cisgenic chestnut

Hypovirulence

Transgenic outcrossing