

Introducing disease resistance into American chestnut: state of the art

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The American Chestnut Foundation

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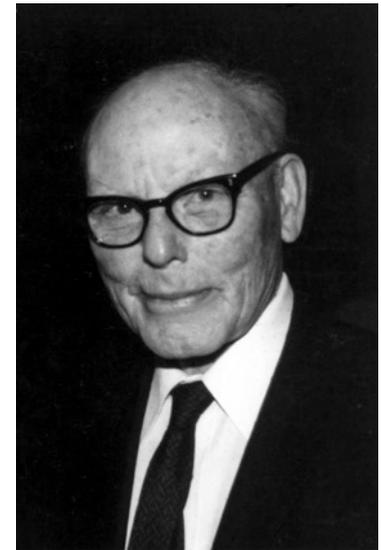
THE
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Backcross breeding predicated on major effect genes for blight resistance

Three generations of backcrossing to recover American chestnut form



Charles Burnham

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



Chinese x American

American

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

F1 x American

1/2
American

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

B1 x American

3/4
American

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

B2 x American

7/8
American

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

B3 x B3

15/16
American

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

B3F2 x B3F2

15/16
American

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

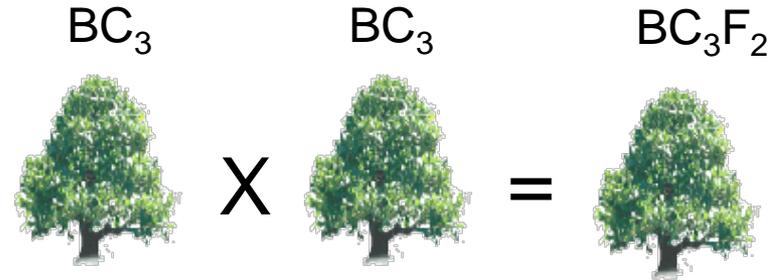
B3F3

15/16
American

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

Two generations of intercrossing to enhance blight resistance

Backcross trees intercrossed to enhance blight resistance



Resistance segregates among the progeny and currently approximately 1 out 150 BC₃F₂ selected



Selecting the 1% in chestnut seed orchards



Eric's six word mission statement: "I plant trees and kill them"

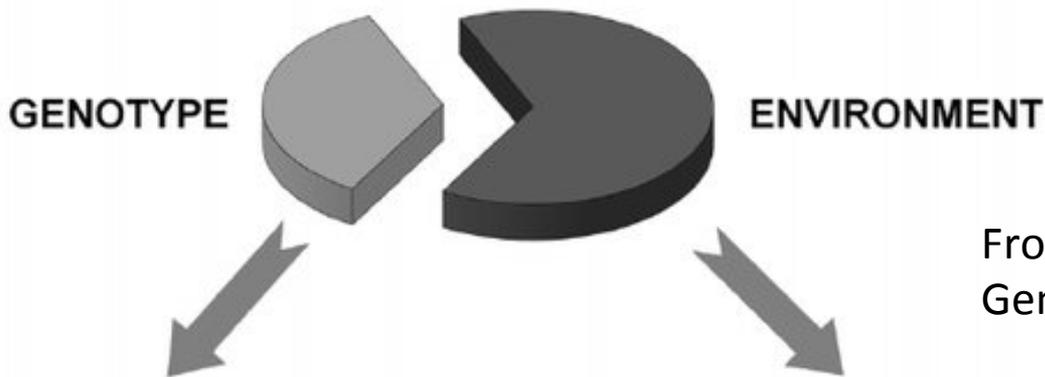
- Selection in 1 of 2 Meadowview BC₃F₂ seed orchards nearly complete
- Approx. 500 trees remaining of 36,000 planted since 2002

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?

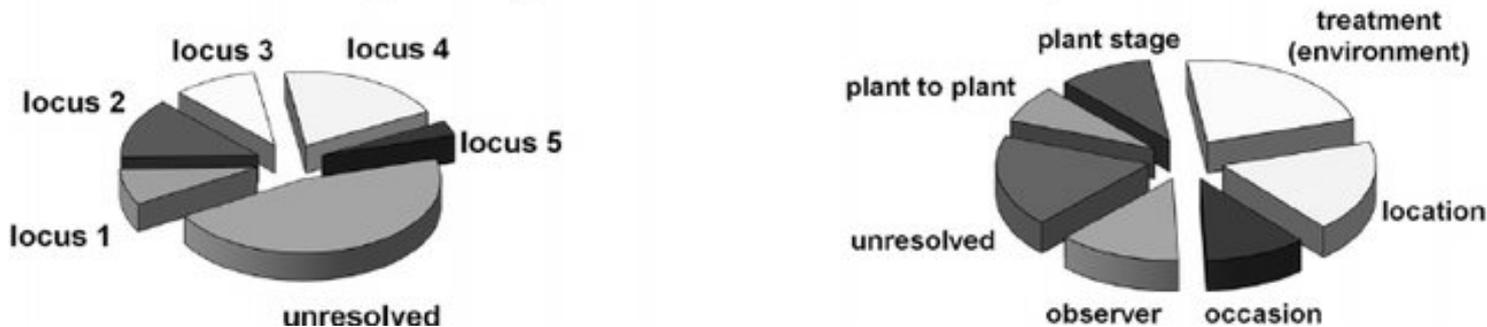
What are the relative effect of genetics and environment on blight resistance?

Phenotype



From King et al. (2010)
Genome 53, 856-868

assigning variance components



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

Measuring blight resistance at age <5: Artificial inoculation and canker severity assessment



Rating 1



Rating 2



Rating 3

- 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?



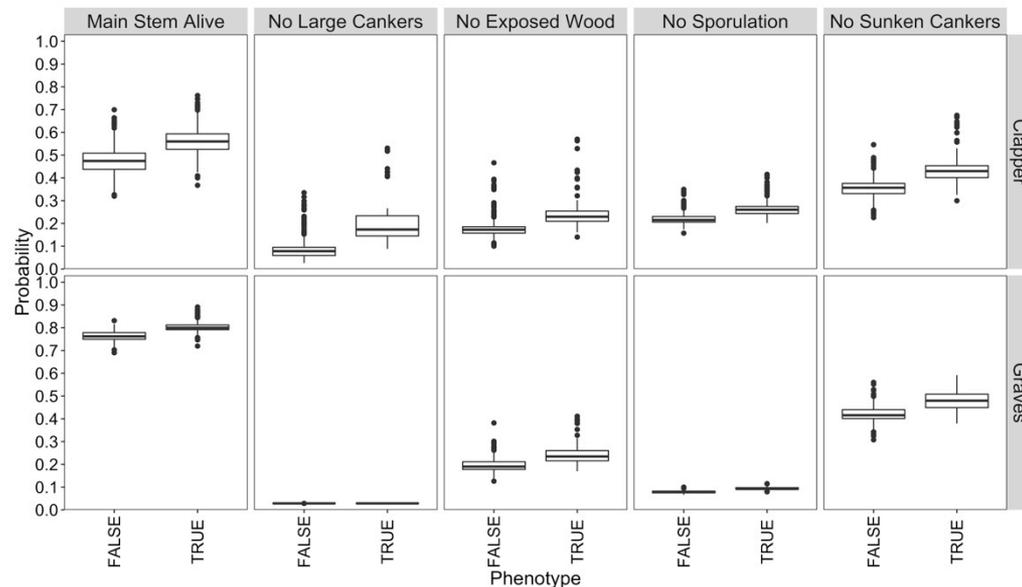
Sporulation?

Phenotype = Age + Block + **Genotype** + Error

Probability of phenotype given genotype

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

	Clapper h^2	Graves h^2
Main stem	0.08±0.04	0.06±0.06
Large cankers	0.25±0.09	0
Sunken cankers	0.09±0.05	0.07±0.05
Exposed wood	0.12±0.06	0.07±0.05
Sporulation	0.07±0.06	0.07±0.05



Parent condition index = sum of blight trait probabilities

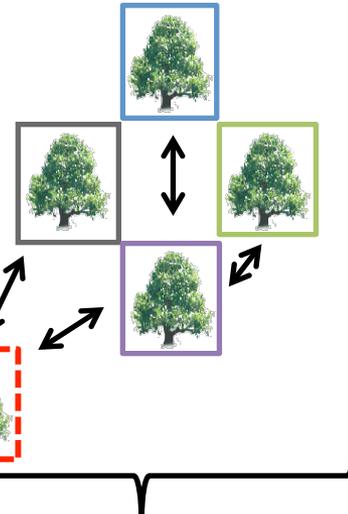
Assessing genetic component of blight resistance with progeny tests

Unselected BC_3F_2 seed orchard

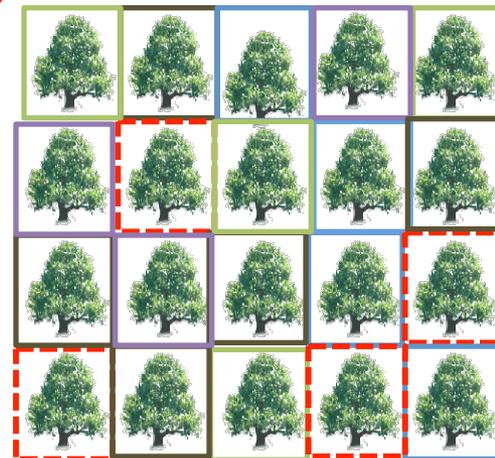
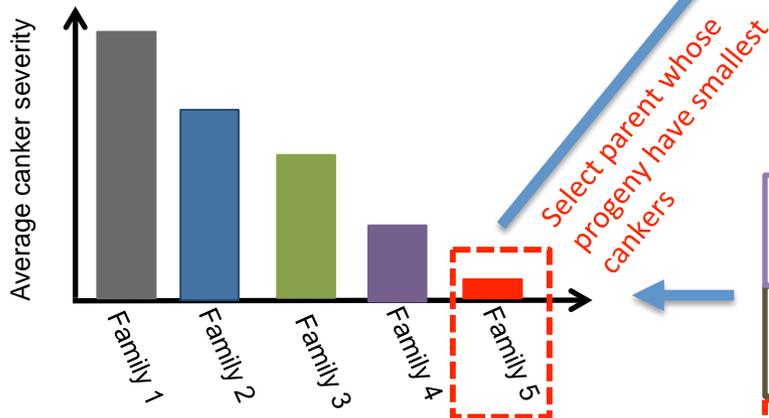


Artificially inoculate & cull susceptible

Partially selected seed orchard



Open-pollination among BC_3F_2 survivors



Artificially inoculate BC_3F_3 progeny from each selection candidate

Average canker severity of progeny of selection candidates moderately heritable



Rating 1



Rating 2



Rating 3

Canker length (cm)

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

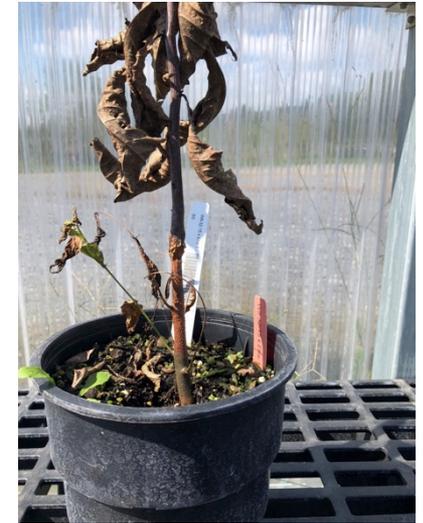


Inoculate first year seedlings with highly virulent strain of of the chestnut blight fungus (Ep155)



Measure

- Days to wilt
- Survival proportion within families 18 weeks post-inoculation

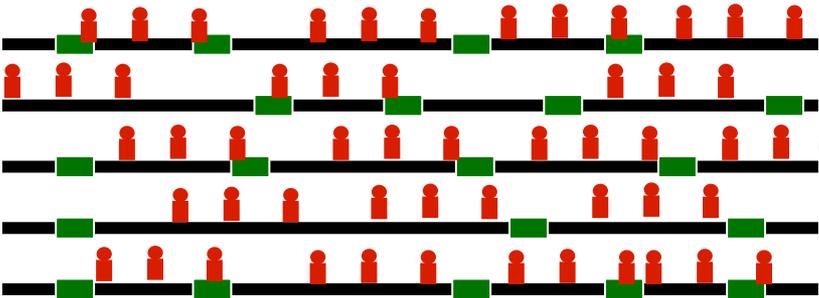


Genomic selection increases speed and accuracy of selection in seed orchards



Genotyping by Qian Zhang and Jason Holliday, Virginia Tech

> 3,000 BC₃F₂ selection candidates genotyped for genomic selection



 DNA marker

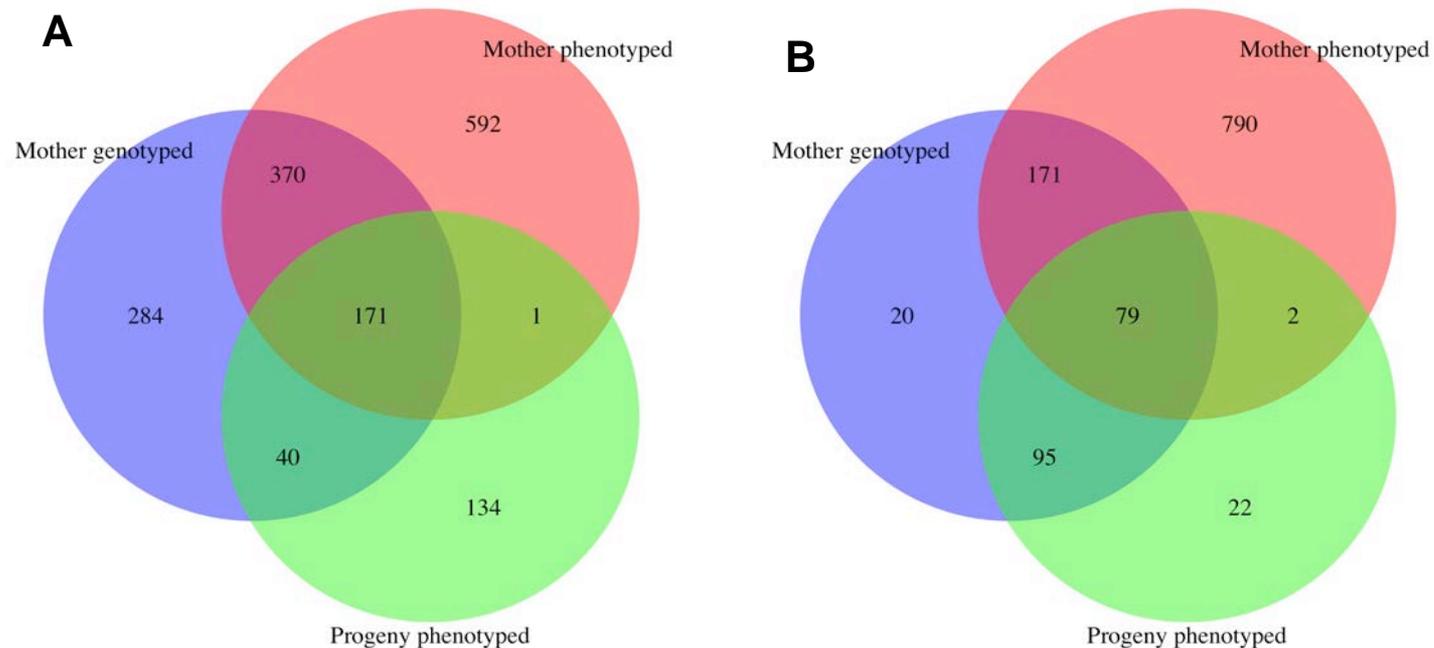
 Gene involved in blight resistance

> 800 BC₃F₃ families progeny tested in orchards or in SSAs



> 2000 BC₃F₂ selection candidates phenotyped for long-term blight resistance

Genomic prediction enables estimation of individual tree resistance and progeny resistance for all selection candidates

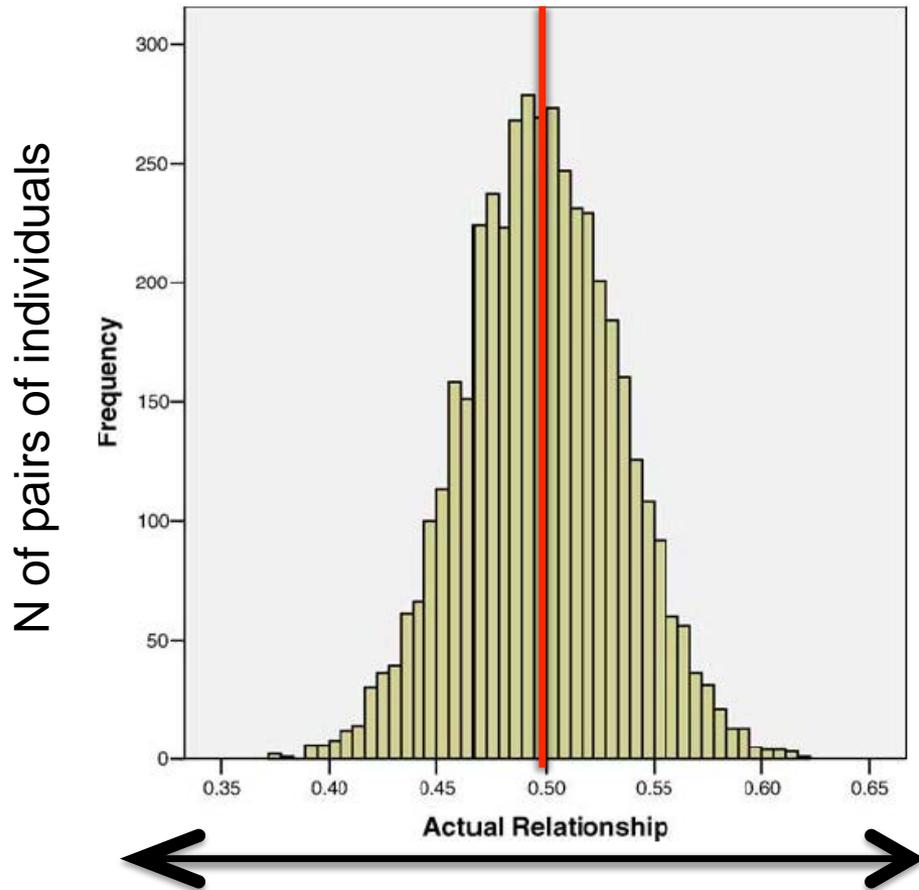


Different subsets of selection candidates phenotyped, progeny tested and genotyped

How genomic prediction works

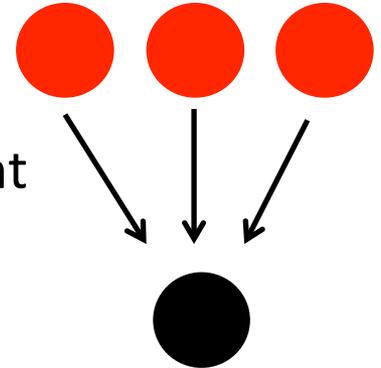
Full sibs expected to share 50% of genome

A better weighted average

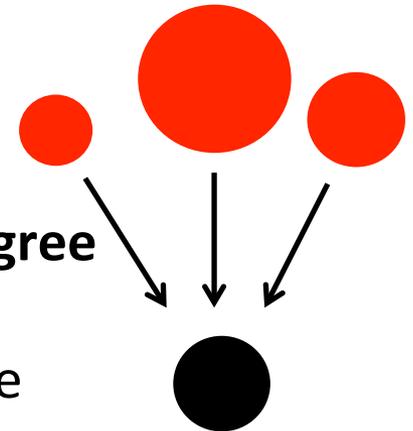


DNA markers trace variation around expected pedigree-relationships

Pedigrees weight sibs equally

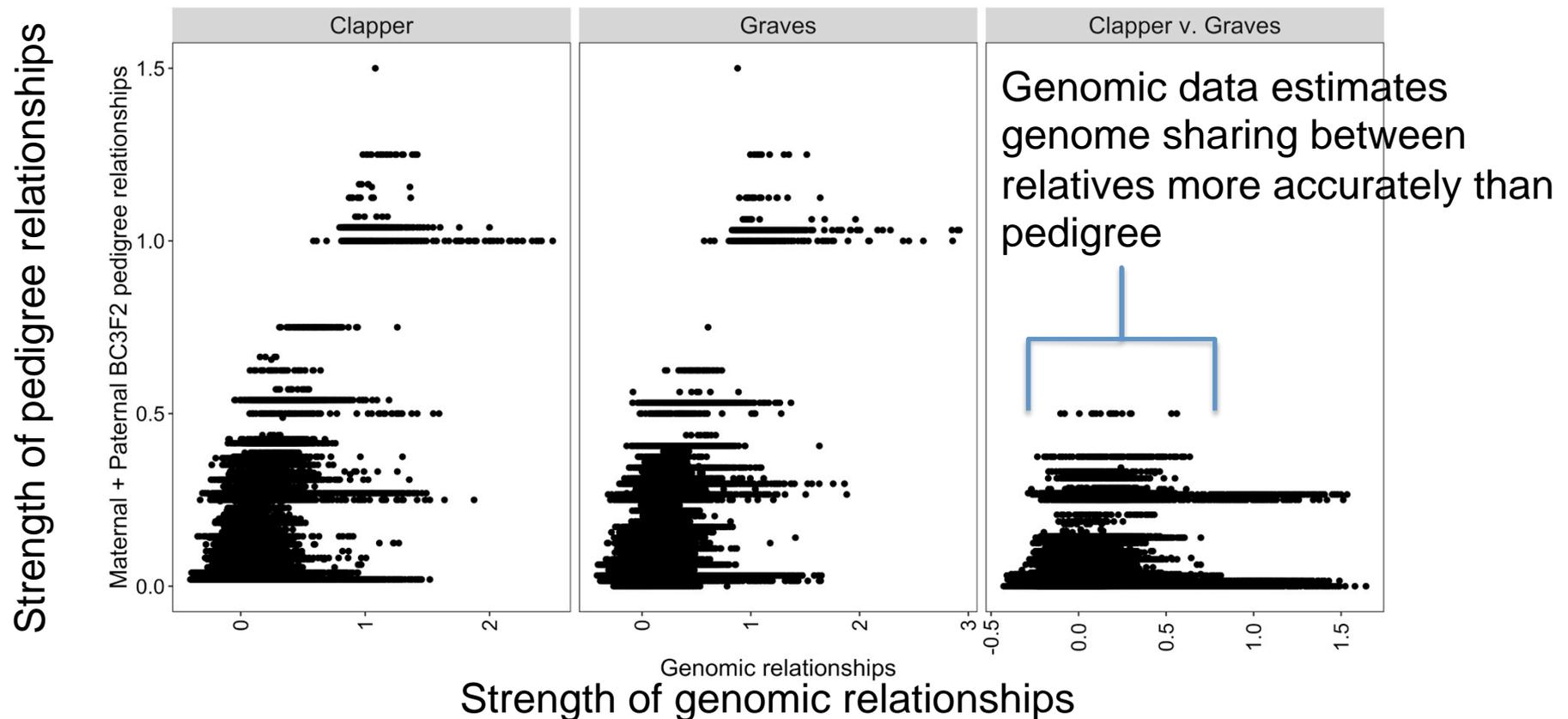


Genomics + pedigree weights sibs in proportion to true relatedness

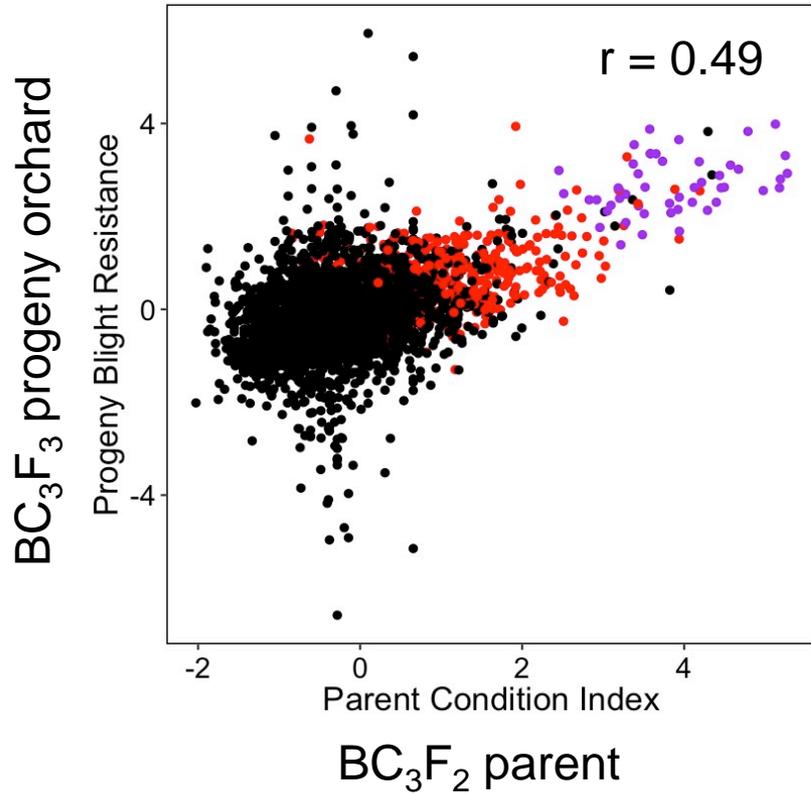


How genomic prediction works

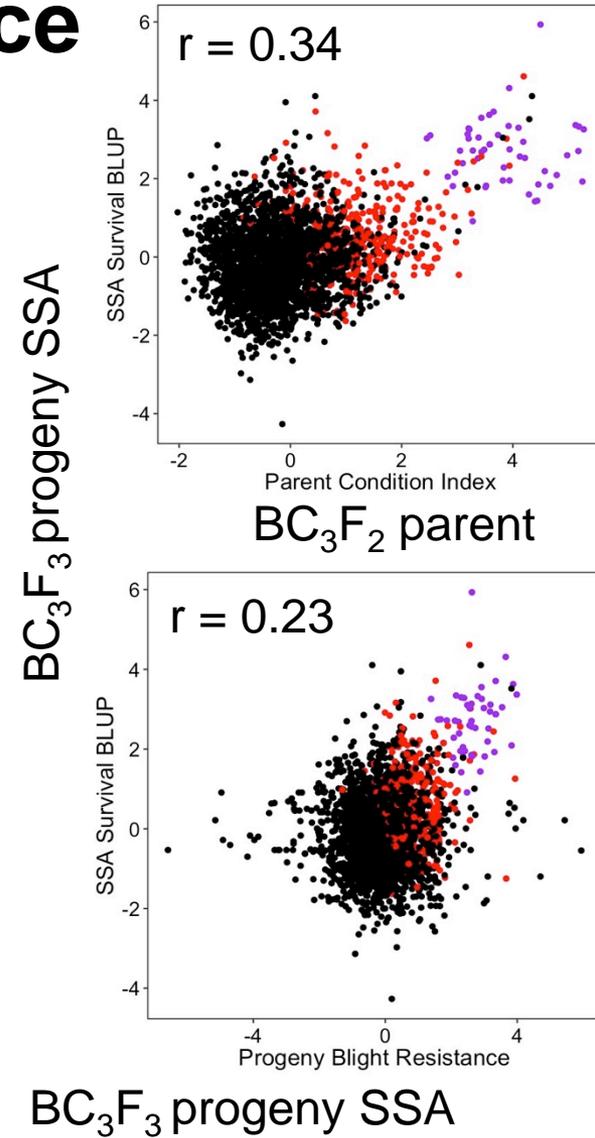
Resistance of non-phenotyped individuals predicted from average of resistance of relatives weighted by strength of relationship



Genomic selections made based on sum of parent and progeny resistance



Blight selection index =
Parent Condition Index
+ $\frac{3}{4}$ (progeny blight resistance)
+ $\frac{1}{4}$ (ssa survival)



Correlations with SSA resistance weaker

Selected backcross (BC_3F_2) trees have a spectrum of intermediate blight resistance



American chestnut



Selected BC_3F_2 with "cruddy bark"



83% American chestnut hybrid with good phenotypic resistance

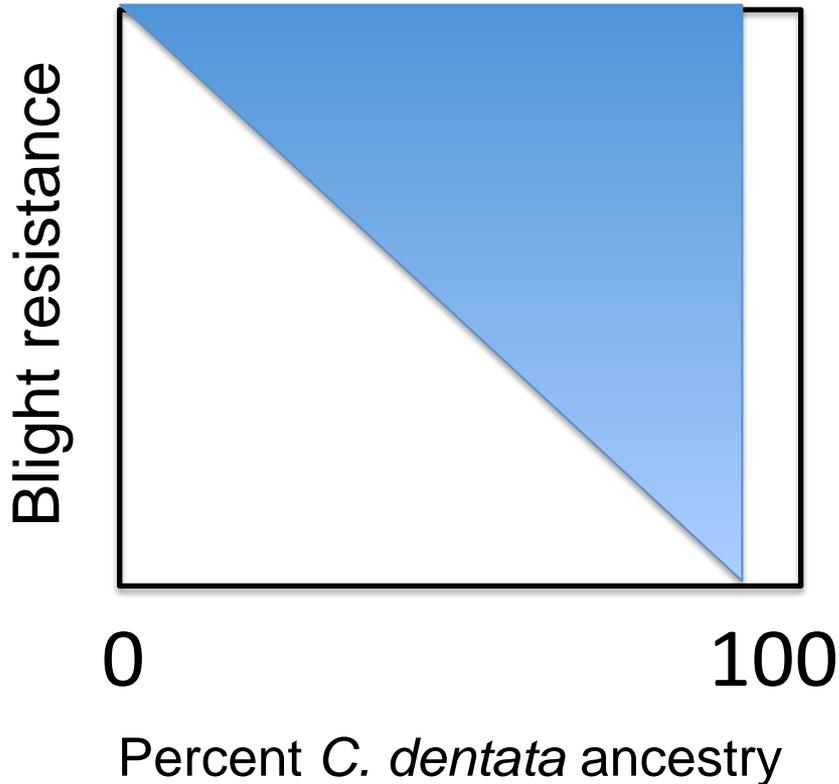


Pseudo-F1 (42% American chestnut)

Is blight resistance controlled by few genes or many genes?

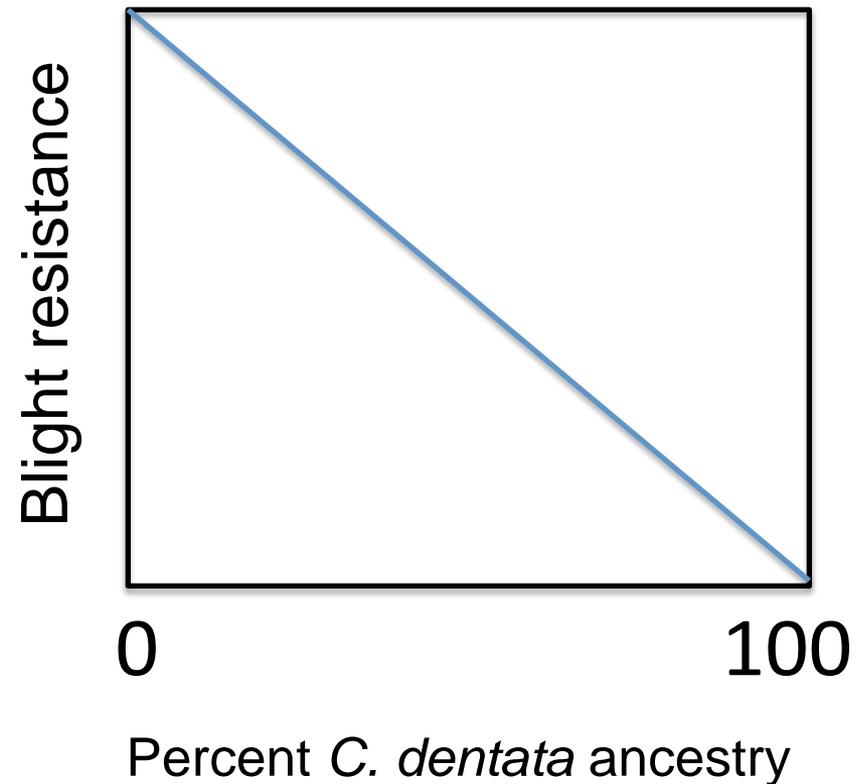
Few genes:

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

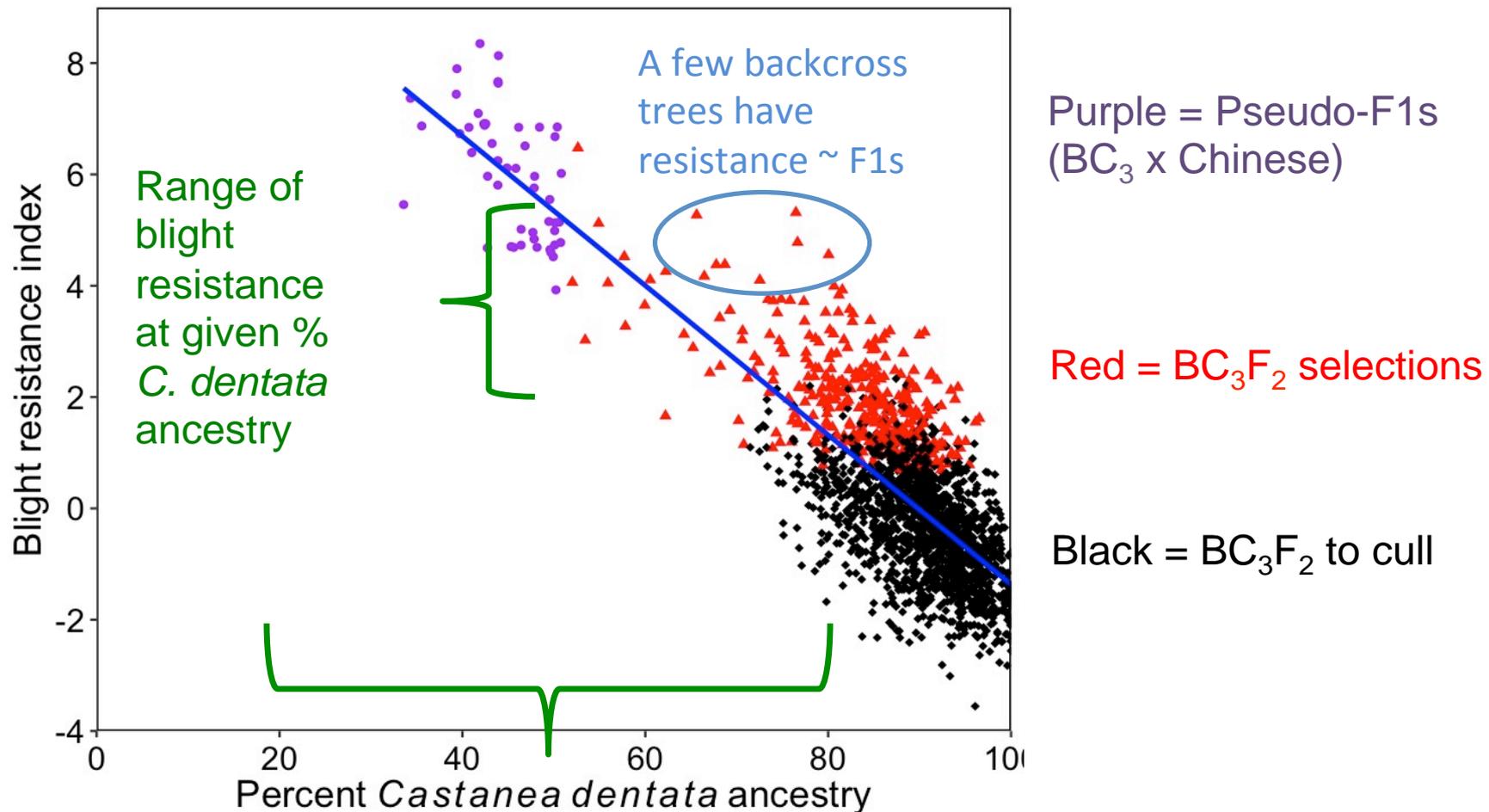


Many genes:

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

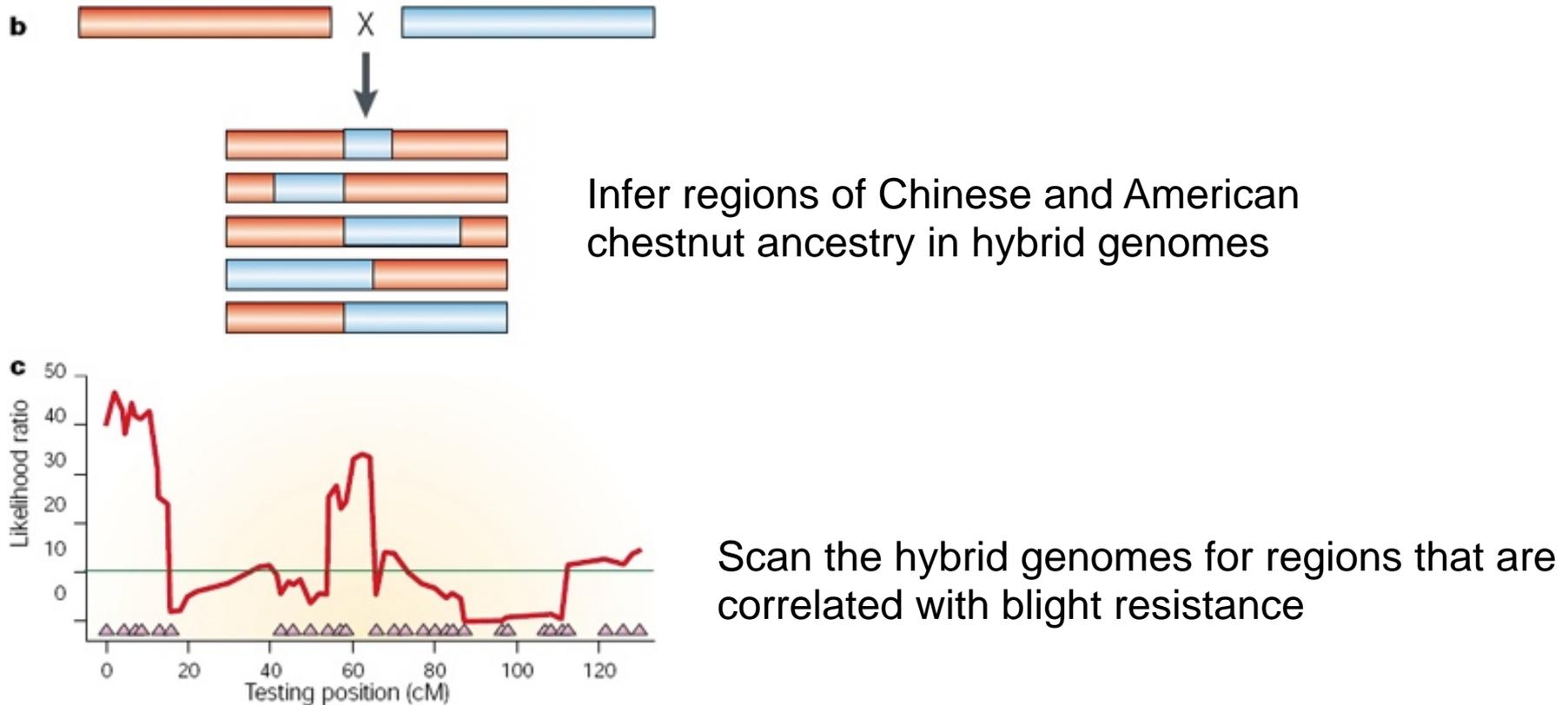


Figure from Mackey, T. F. Quantitative trait loci in *Drosophila*. *Nature Reviews Genetics* 2, 13 (2001)

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

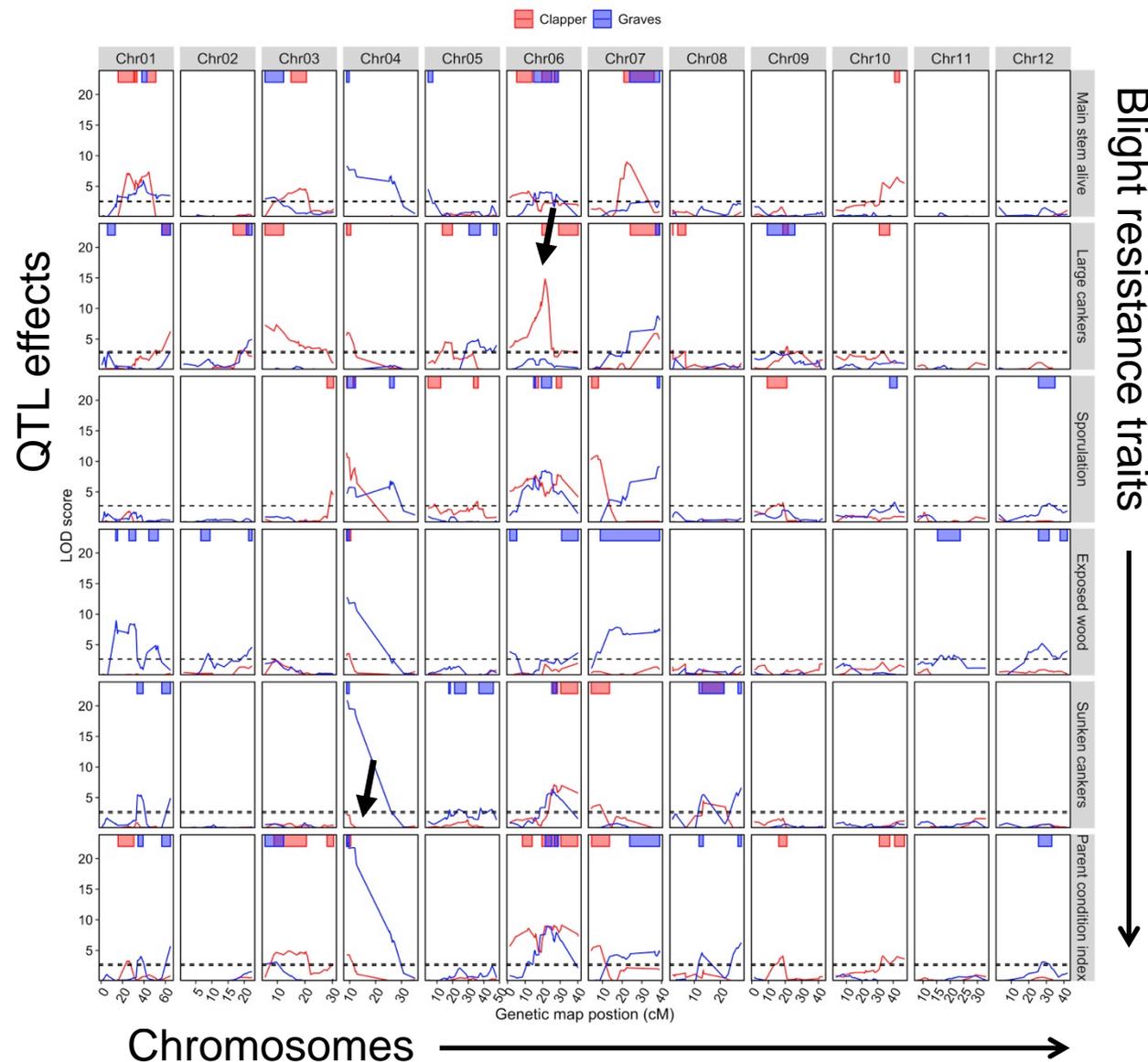
Min QTL interval = 0.7 Mb

Avg. trait variance explained by single QTLs = 3%

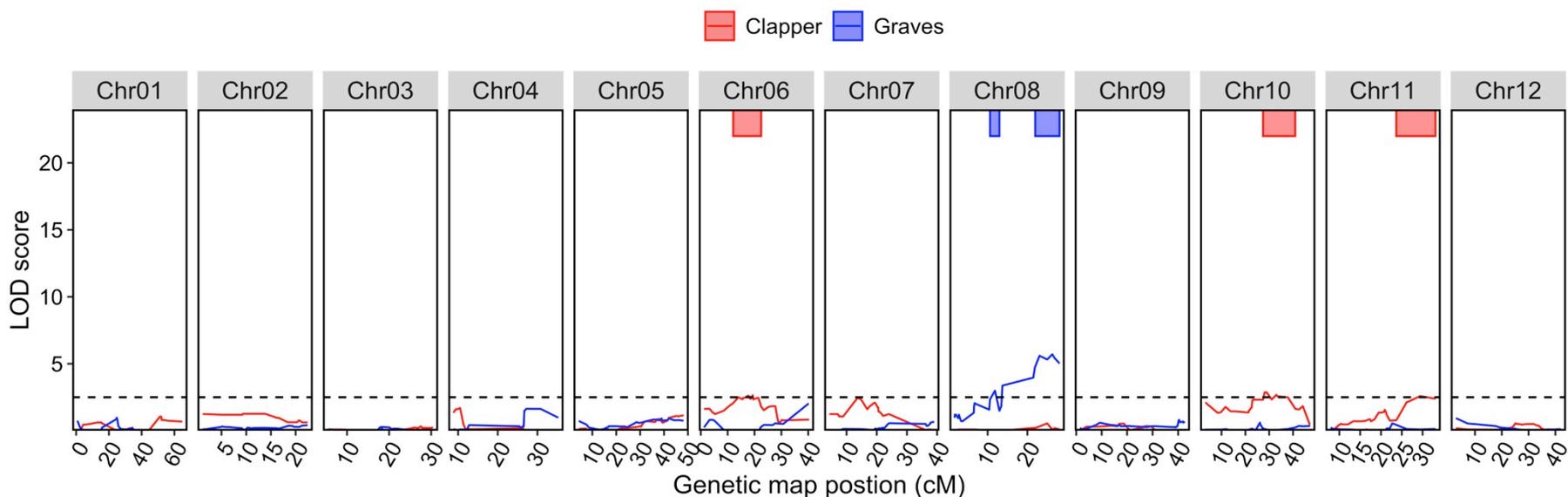
Total trait variance explained by all QTL = 2% to 57% depending on trait & population

Variance explained by largest single QTL = 13%

Clapper and Graves have unique and shared QTLs



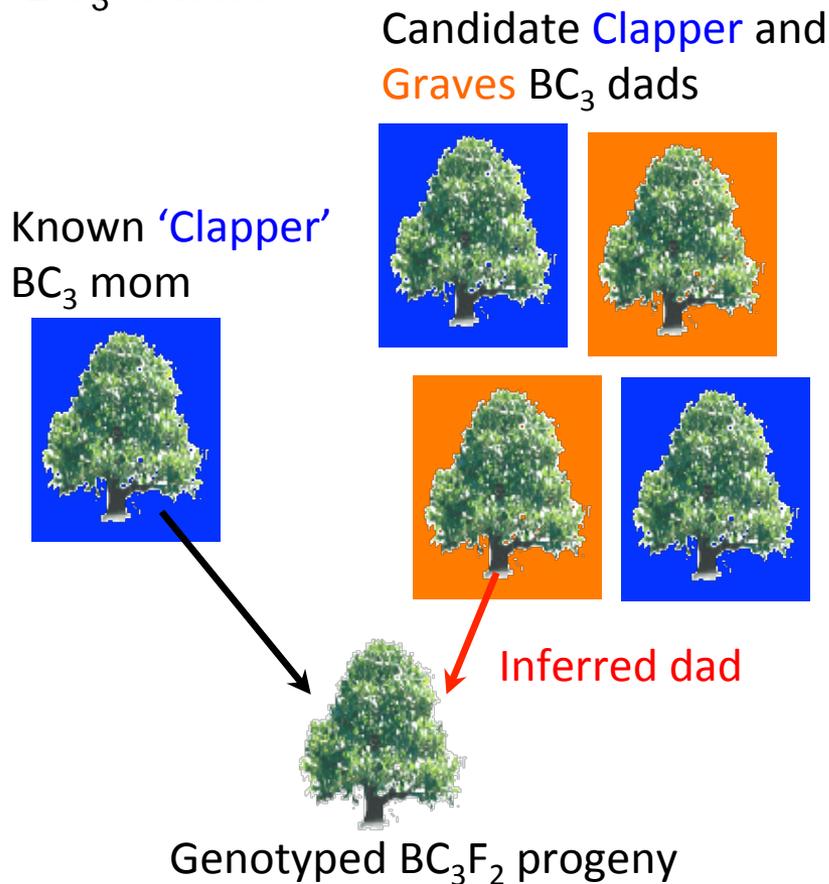
Subset of genomic regions associated with blight phenotypes of BC₃F₂ parents also associated with average canker severity of BC₃F₃ progeny



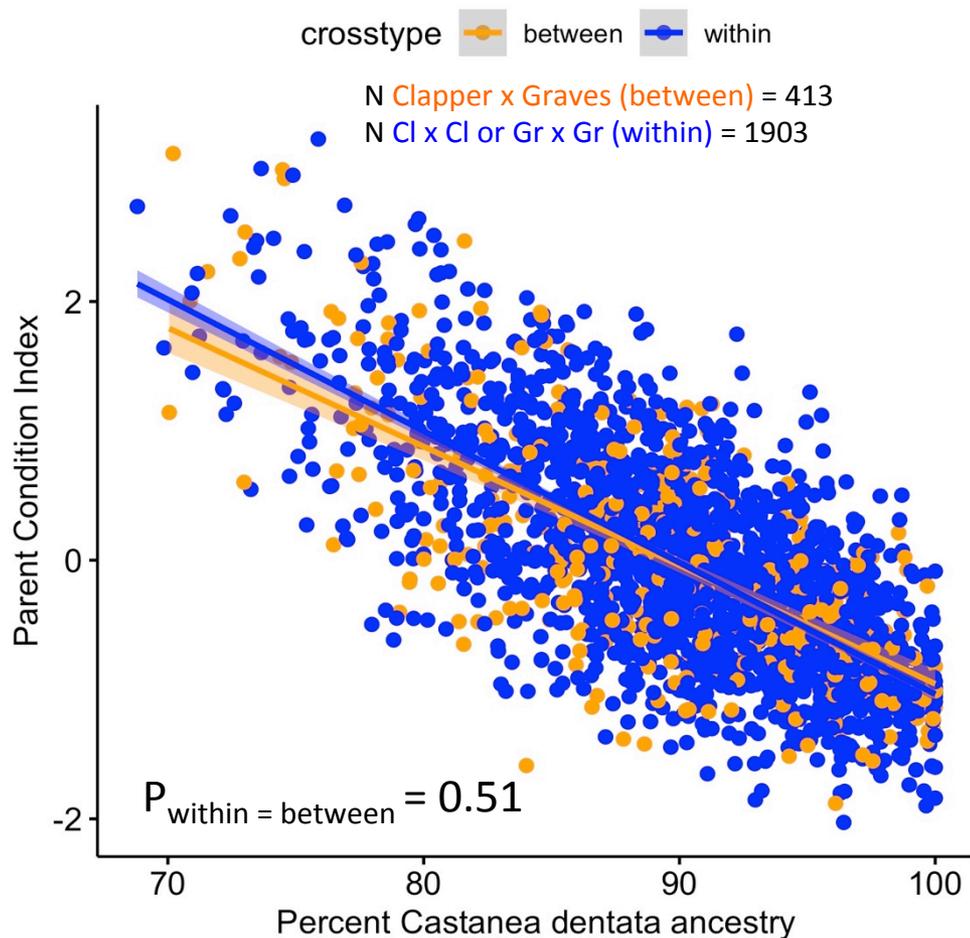
- QTLs explain 15.3% and 17.5% of the variation in BC₃F₃ progeny canker severity in 'Clapper' and 'Graves' populations, respectively.
- Likely additional QTLs, but population of BC₃F₂ mothers whose progeny have been screened for blight resistance too small to detect additional QTL.

No evidence that crossing **Clapper** and **Graves** enhances resistance of BC_3F_2 s

Infer paternity of BC_3F_2 with genotypes of BC_3F_2 s and candidate BC_3 fathers



Paternity analysis by Fred Hebard

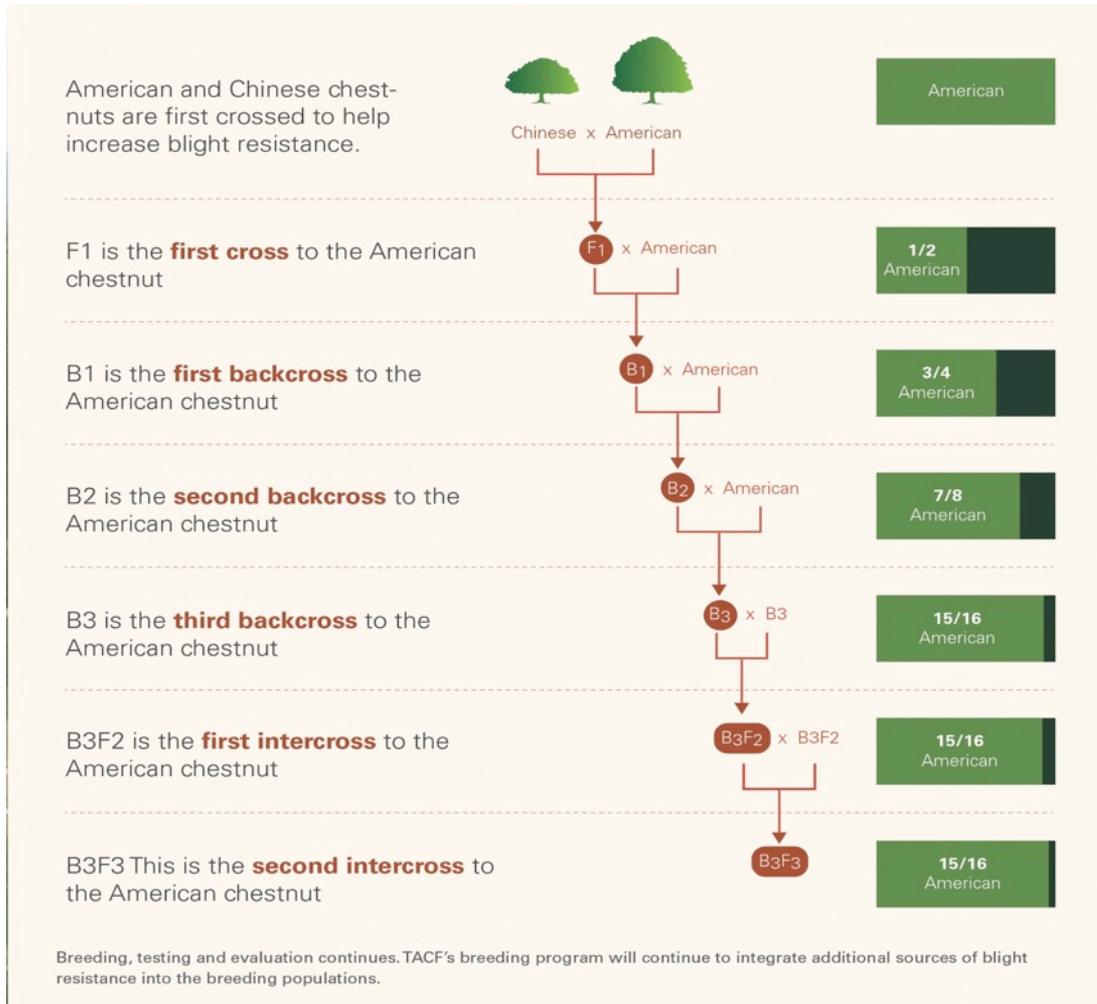


Breeding: The path forward

- Increase resistance by increasing Chinese chestnut ancestry
 - As much as necessary as little as possible
- Cross inferior backcross lines with OxO
- Moderate expectations



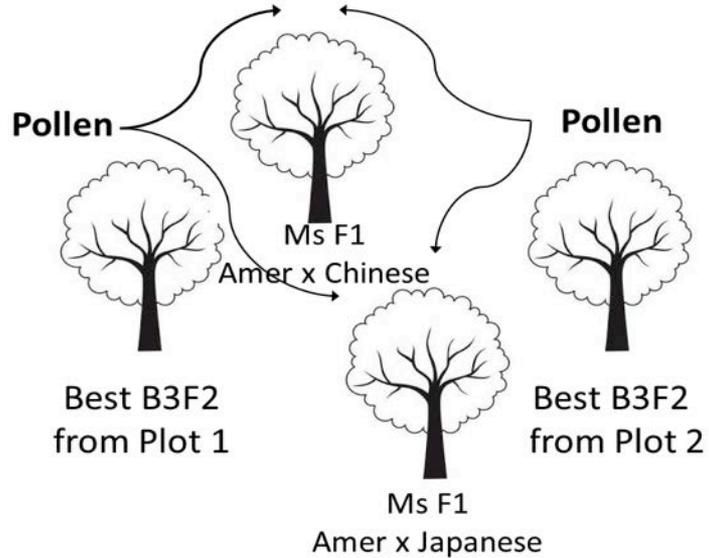
Finding a balance between blight resistance and American traits in traditional breeding



Increase blight resistance by intercrossing BC_3F_2 s with F_1 s and BC_1 s

Increase stringency of selection at BC_3/BC_4

Plant male sterile F₁s in BC₃F₂ seed orchards to generate “Better BC1s” with enhanced resistance



American x Chinese

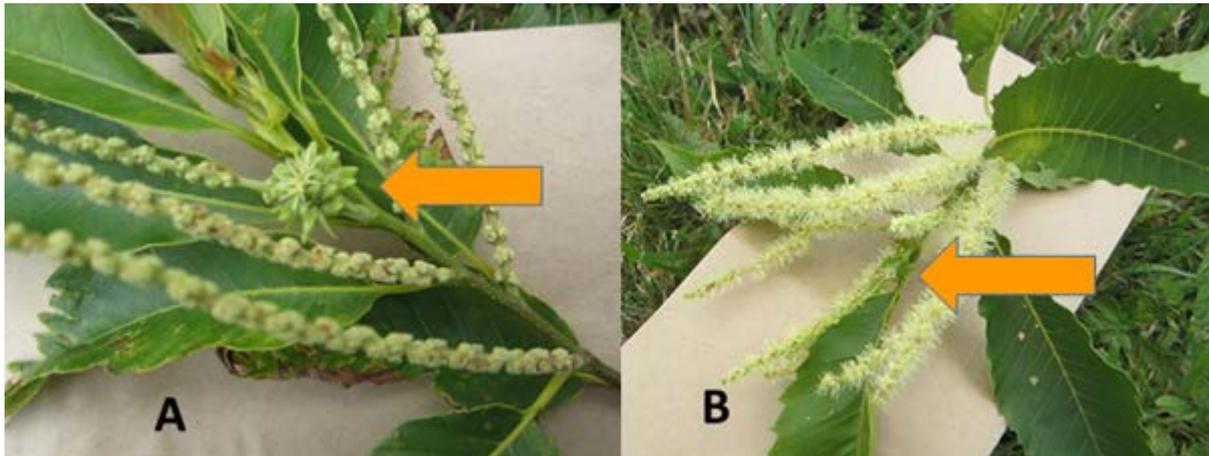
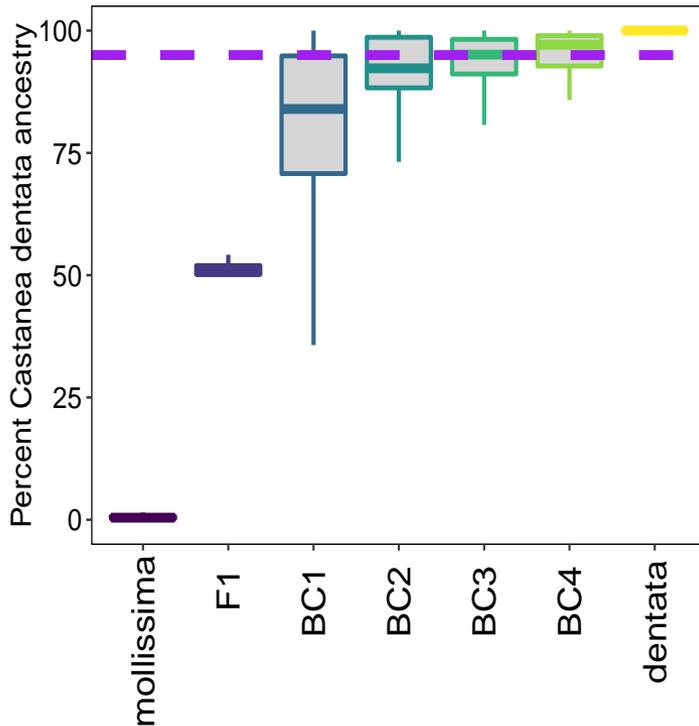


Figure and photos by Paul Sisco

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



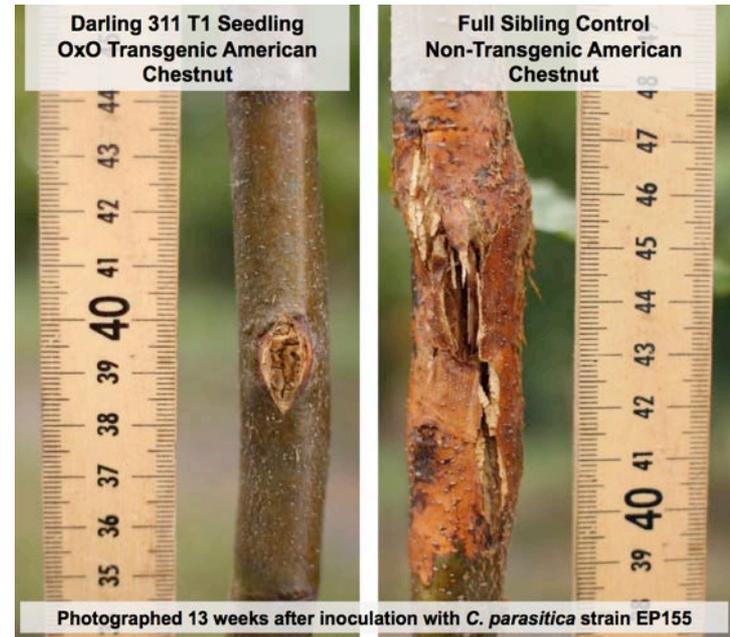
251 of 521 trees candidate backcross selections genotyped to date have >95% American chestnut ancestry and are likely to have inferior blight resistance

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

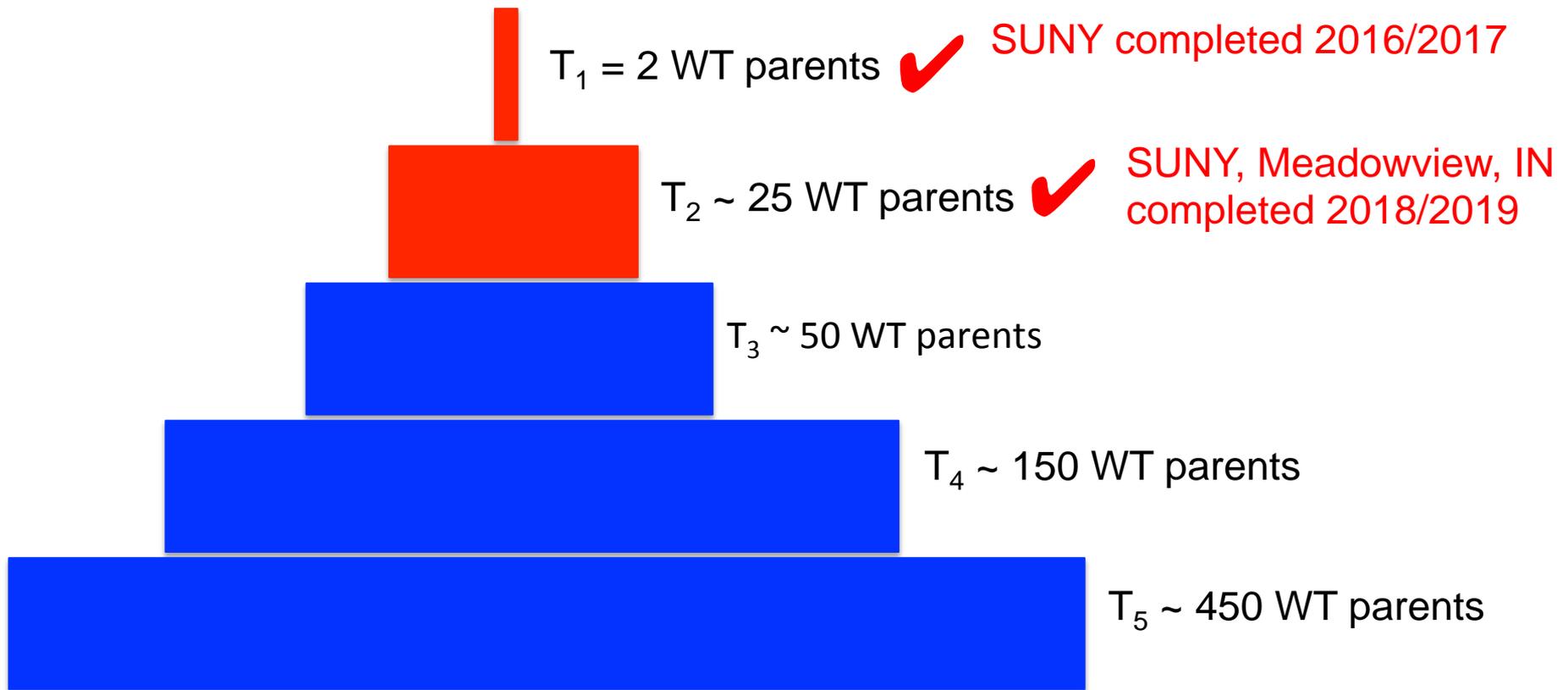


Apply transgenic-OxO pollen to wild-type trees



50% of progeny expected to inherit OxO

Outcross O_xO to wild-type trees over five generations 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

Location	N mother trees	N seeds	N (%) OxO positive
SUNY	20 WT,	893	337* (44%)
Meadowview	3 LSA, 2 BC ₃ F ₂	519	219 (42%)
Purdue	10 WT	638	303 (44%)

* 795 nuts tested for OxO to date

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)



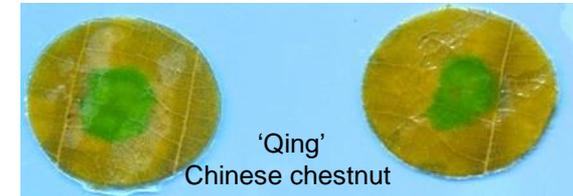
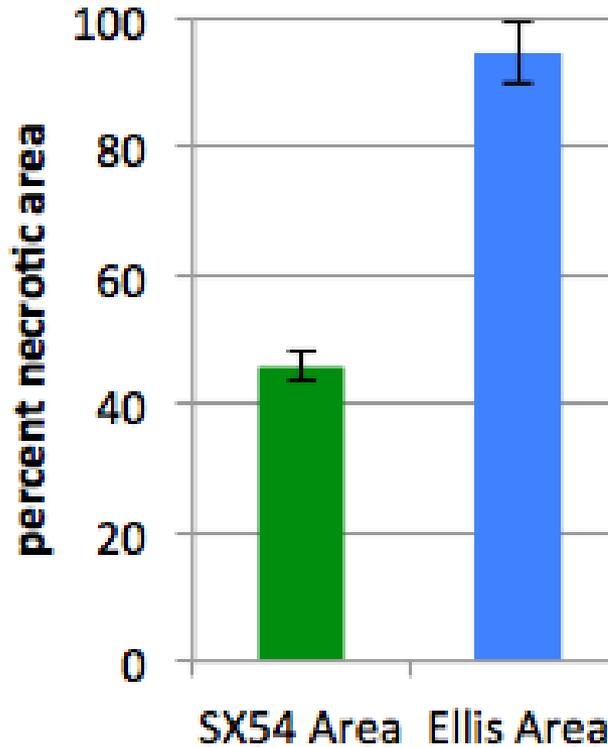
OA leaf disk assay (no fungus)

Green – living tissues
Brown – dead tissues

Lower bar = more tolerant



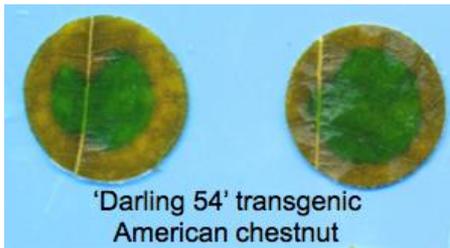
Oxalic acid 50mM Soak



Chinese chestnut has no Oxalate oxidase, so...

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

OA tolerance trait



N=10

Mycelial fans form more rapidly in American chestnut relative to Chinese chestnut, which is resistant to blight

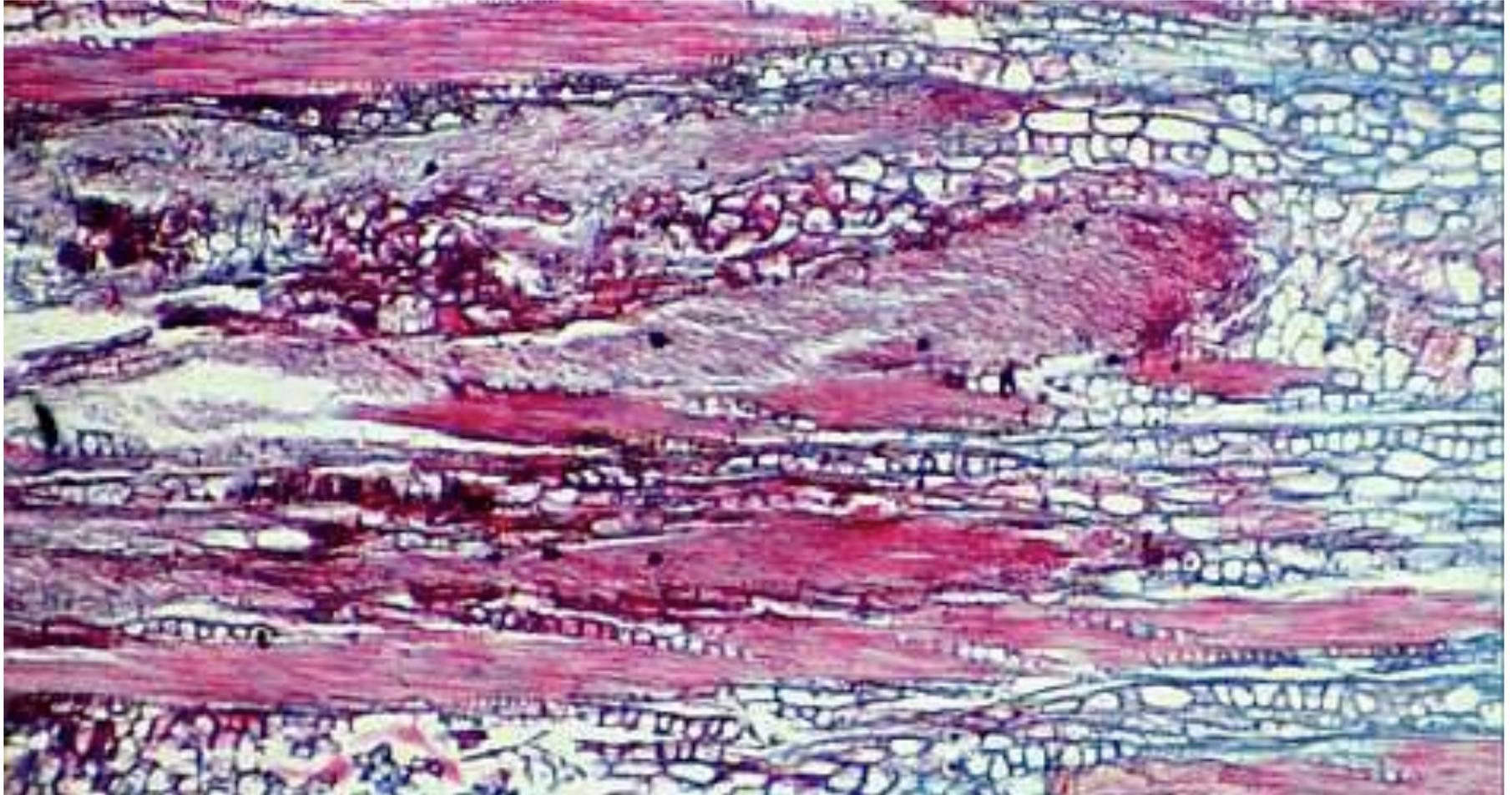


Photo: Fred Hebard

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

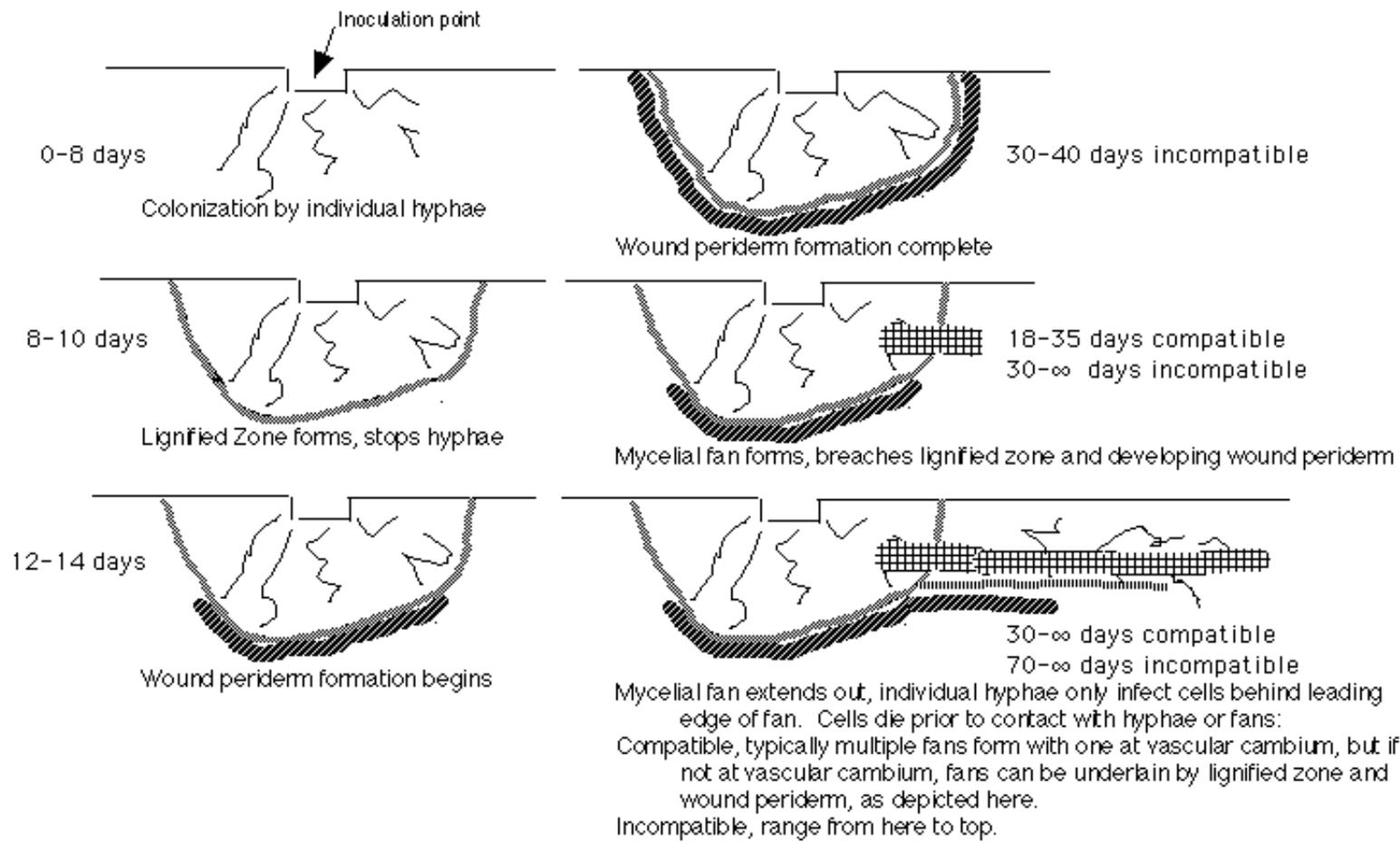


Figure: Fred Hebard

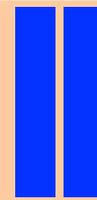
Chinese chestnut forms thicker periderm faster than American chestnut

Strategy for discovering additional candidate genes for blight resistance

Step 1: Assemble chestnut reference genomes

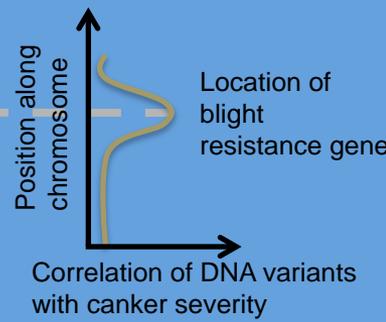
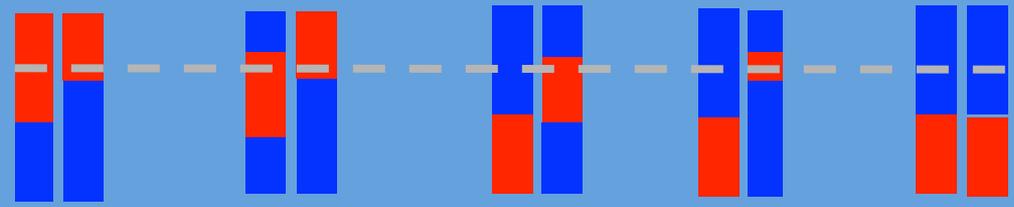


Chinese chestnut source of resistance

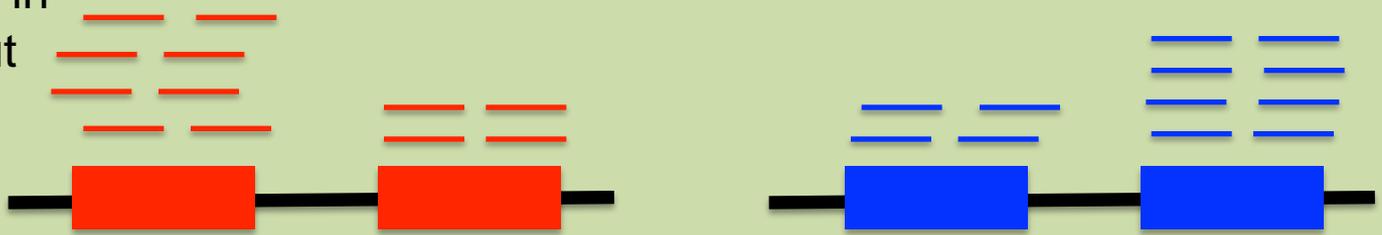


American chestnut

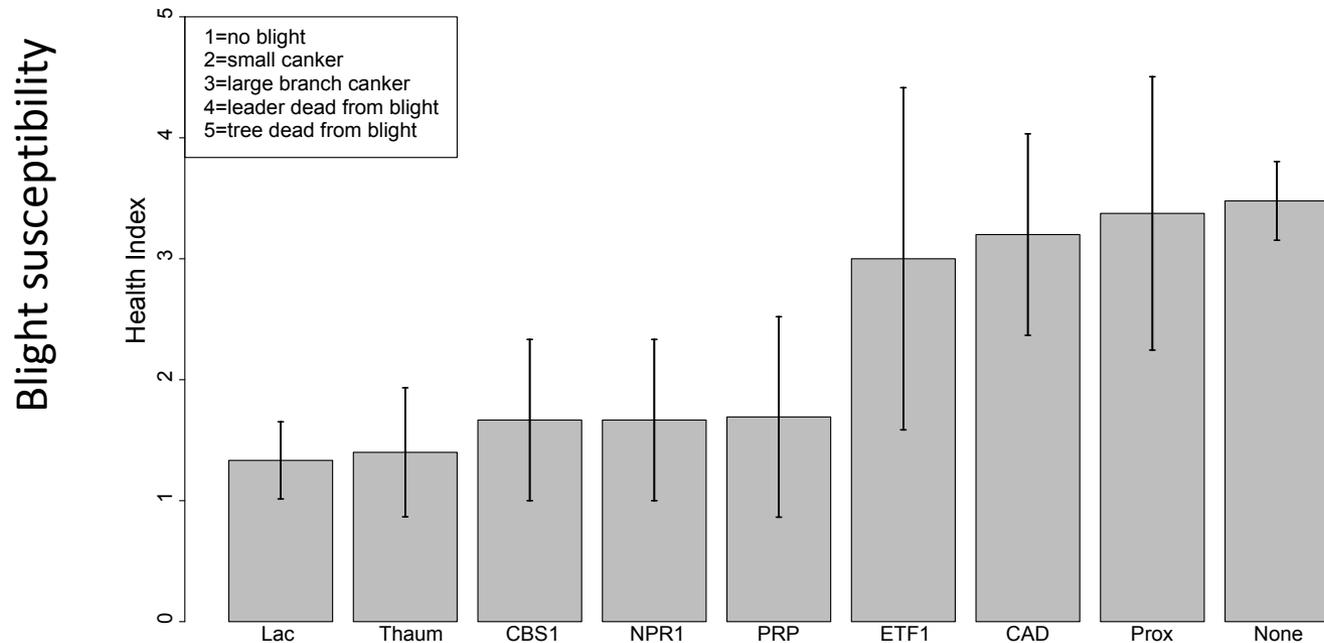
Step 2: Scan hybrid genomes for regions correlated with blight tolerance



Step 3: Compare gene expression in Chinese chestnut and American chestnut stems after blight infection



Chinese chestnut cisgenes enhance blight resistance in American chestnut



Transgenic American chestnuts with candidate genes from Chinese chestnut

wildtype *dentata*



State University of New York College of Environmental Science and Forestry



VIRGINIA TECH™

Forest Health Initiative

Exploring Biotechnology to Protect Forest Health

CRISPR applications: Adjacent insertion of multiple resistance genes to simplify inheritance of polygenic resistance

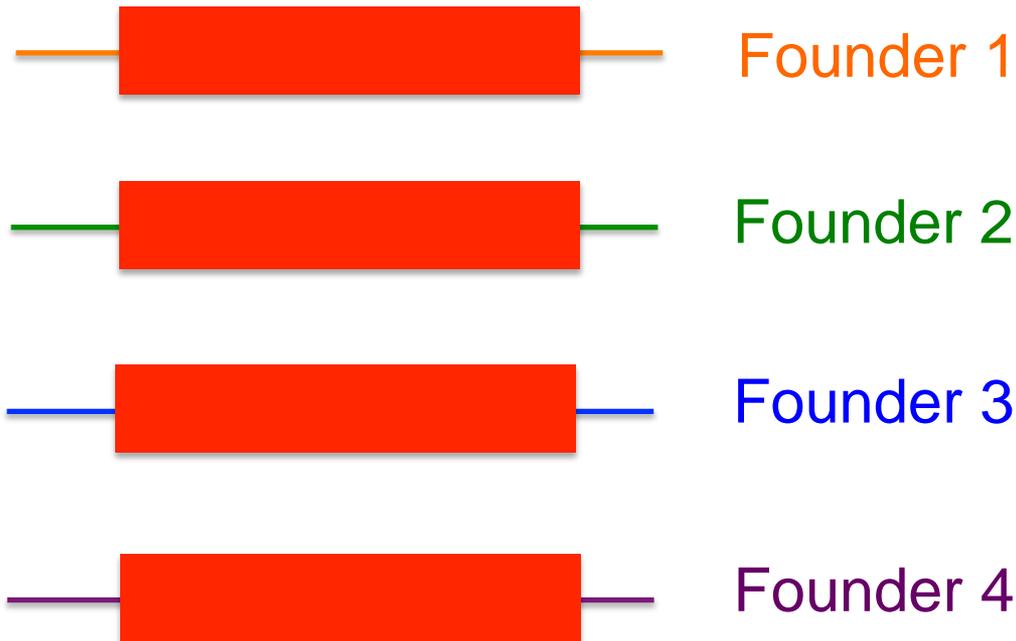
Blight
resistance
gene A

Blight
resistance
gene B

Root rot
resistance
gene



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?

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

Additional breeding to combine blight and PRR resistance

PRR resistant
BC3-F2
selections

Blight resistant transgenic
outcross progeny

Intercross to increase PRR
resistance



X



X



X

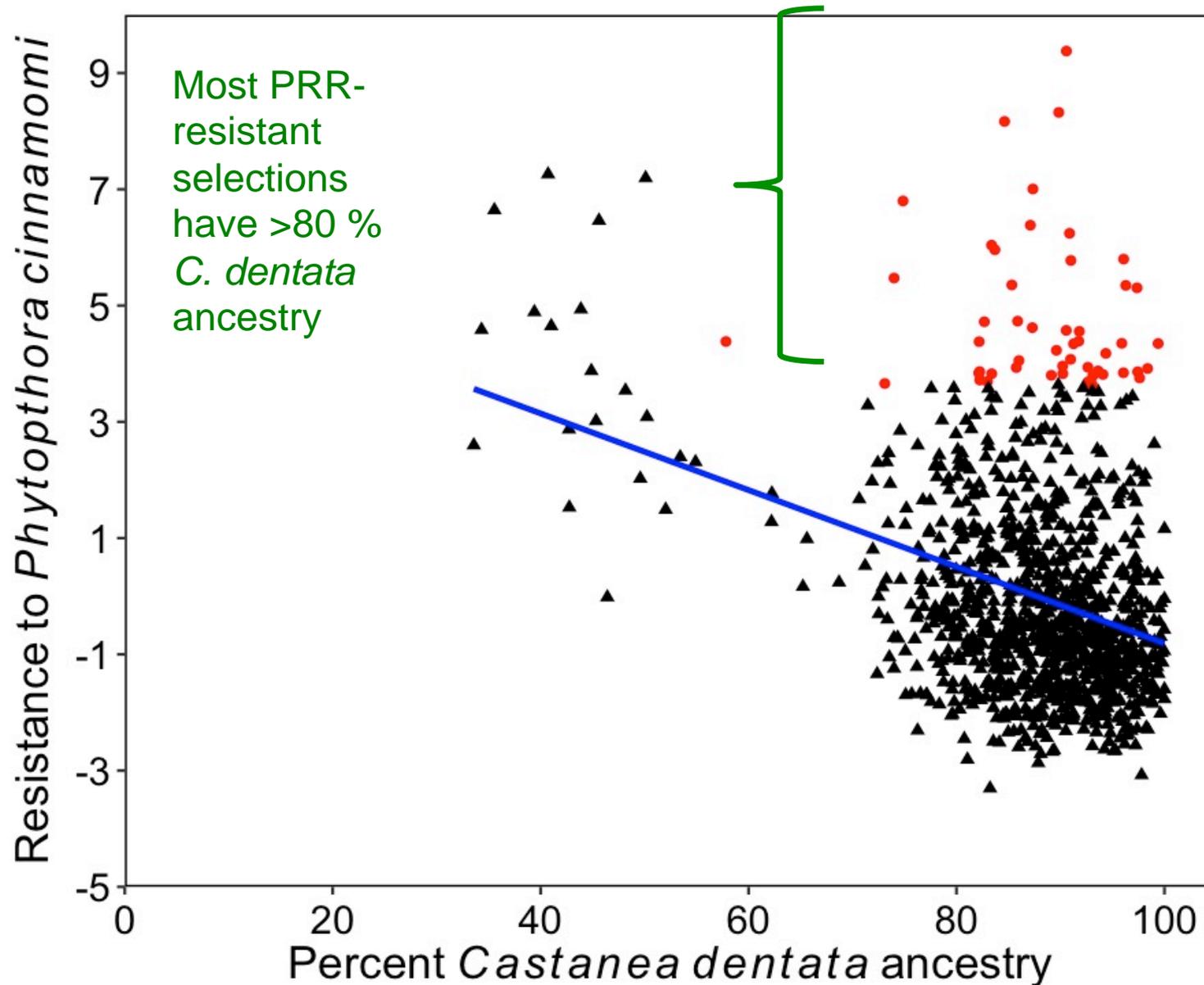


Select for PRR
resistance and
deploy

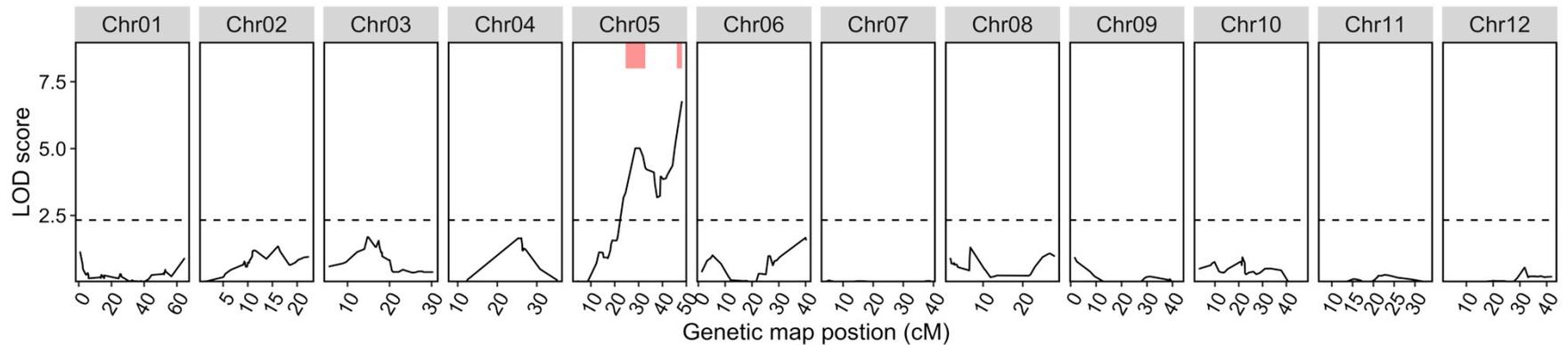


2 generations of
breeding and selection

Possible to generate hybrids with > 80% American chestnut ancestry AND high resistance to *P. cinnamomi*

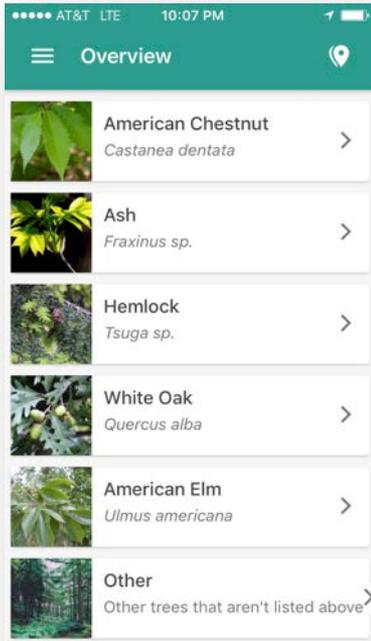


Fewer, larger effect QTL associated with resistance to *Phytophthora cinnamomi*



American chestnut backcross trees that survived inoculation have partial resistance to *P. cinnamomi*

Germplasm conservation for diversifying 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

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

3BUR: Breeding, Biotecchnology, and Biocontrol United for Restoration

Conservation of backcross lines

Selection for blight tolerance

Backcross breeding



Selection for Phytophthora resistance



Germplasm conservation



Hypovirulence



Transgenic outcrossing



Genomics



Genomic selection

Discovering genes for resistance

Marker-assisted introgression

Cisgenic chestnut

