Transgenic American chestnuts for potential forest restoration:

Scientific successes, regulatory challenges

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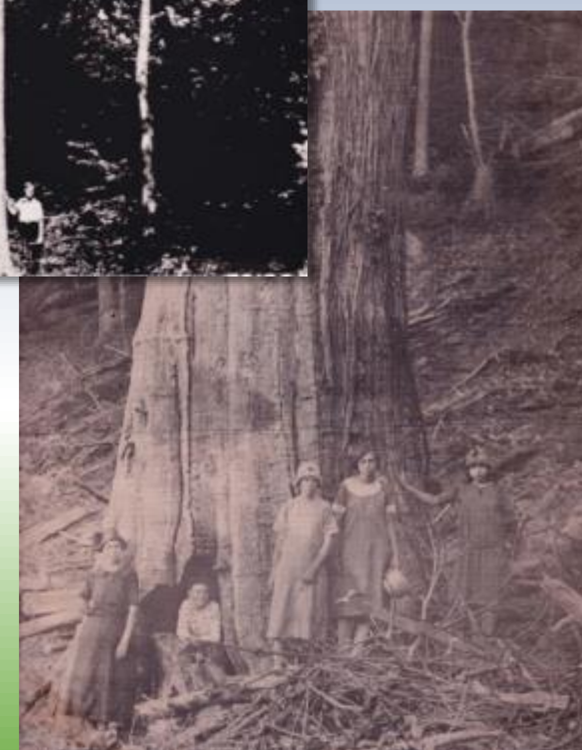
Giants of the Eastern Forests

ESF



Food



Culture

Chestnuts roasting on an open fire,
The Christmas Song
(Torme and Wells, 1946)

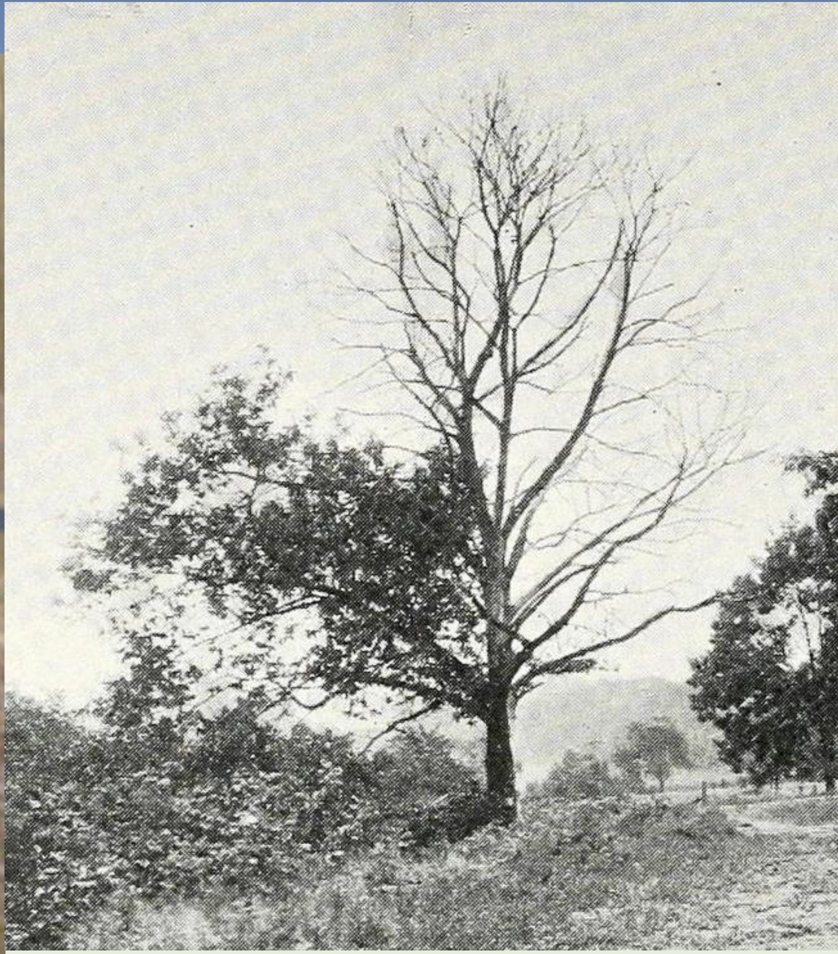
Wood products


Wildlife






Chestnut Blight



Threatened, but not endangered: *Functionally* extinct

Options for responding to blight: Which involve risks?

- Do nothing (active decision with consequences!)
- Breeding (Asian chestnuts tolerate blight)
  - Plant hybrids
  - Backcrossing
- Mutagenesis
- Biocontrol / Hypovirulence

Not regulated

- Genetic engineering

Regulated

Consider:

GE risks or perceptions *relative to traditional methods*

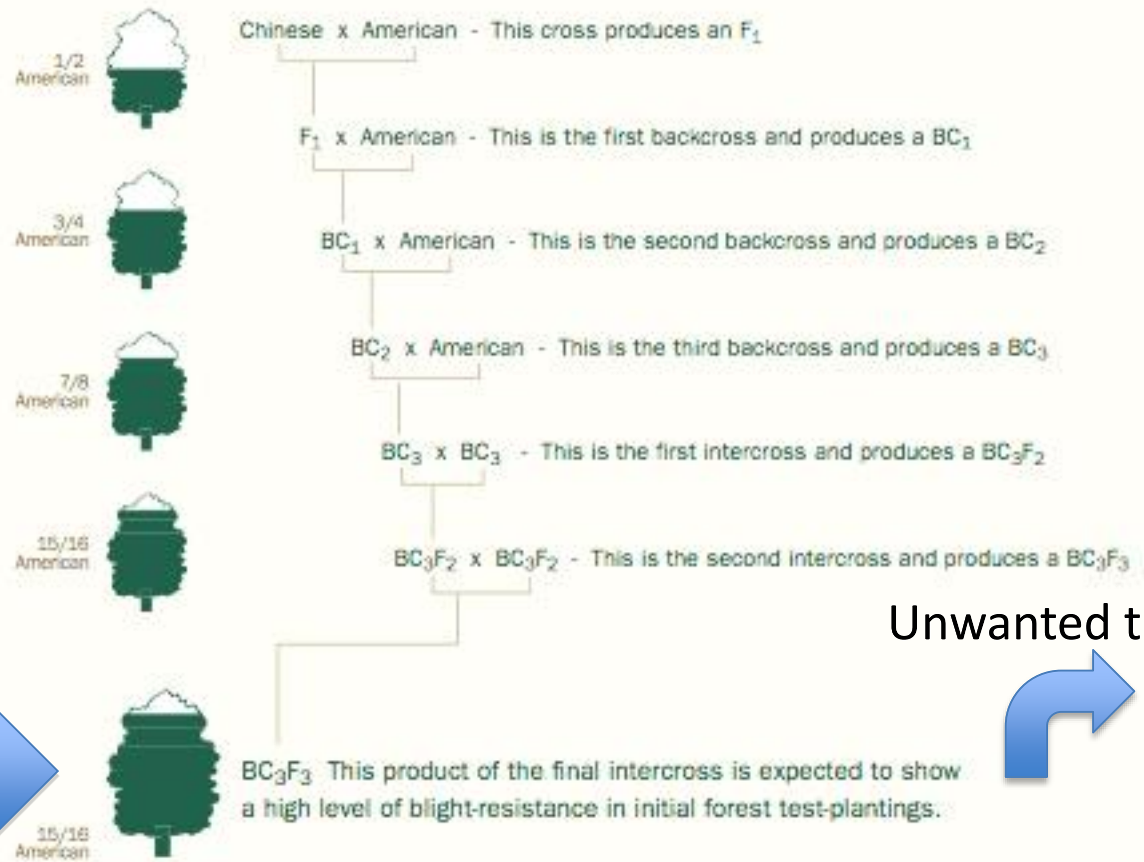
Figure 1.

THE AMERICAN CHESTNUT FOUNDATION BACKCROSS BREEDING PROGRAM

With each cross, additional American chestnut characteristics are regained. Only at the final cross, however, does blight resistance approach that of the Chinese parent

~40,000 CC genes + ~40,000 AC genes

TACF Meadowview
Farm, VA
Dr. Fred Hebard
(started 1983)



Goal is for 1/16 Chinese chestnut genome to contain the 3 or more blight resistance genes

Unwanted traits

Note: In each step, the Backcross is selected for resistance through the process of inoculation and for American charcteristics by visual observation.

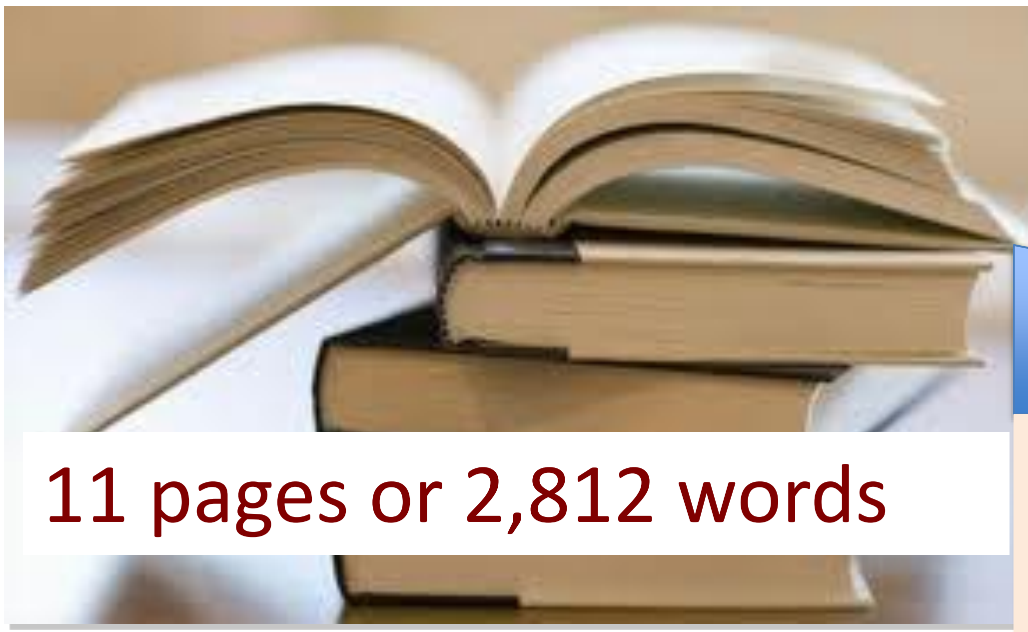
~93% American chestnut

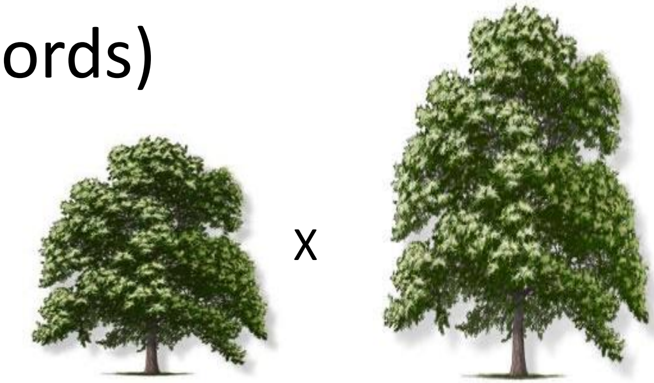
Breeding & Transgenics:

(Both viable options & both have advantages & disadvantages)

Chestnut has ~ 40,000 gene pairs (words)

1/16 Chinese chestnut genes:





Making very small changes, adding only 2 genes/words

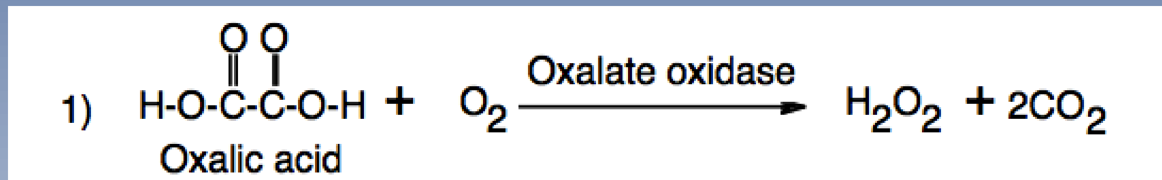
It was very exciting at that season to roam the then boundless ^blight resistant^ chestnut woods of Lincoln, …

Henry David Thoreau, "Walden: or Life in the Woods," 1899

> 99.999% American chestnut

Oxalate oxidase (OxO) from wheat

Detoxifies oxalate (oxalic acid)



-***Doesn’t kill fungus***: Reduces the chance of the fungus developing resistance to oxalate oxidase, eases concerns about unnecessarily killing pests

-Easily detected to ID our transgenic trees

-Also naturally found in:

Other cereal grains

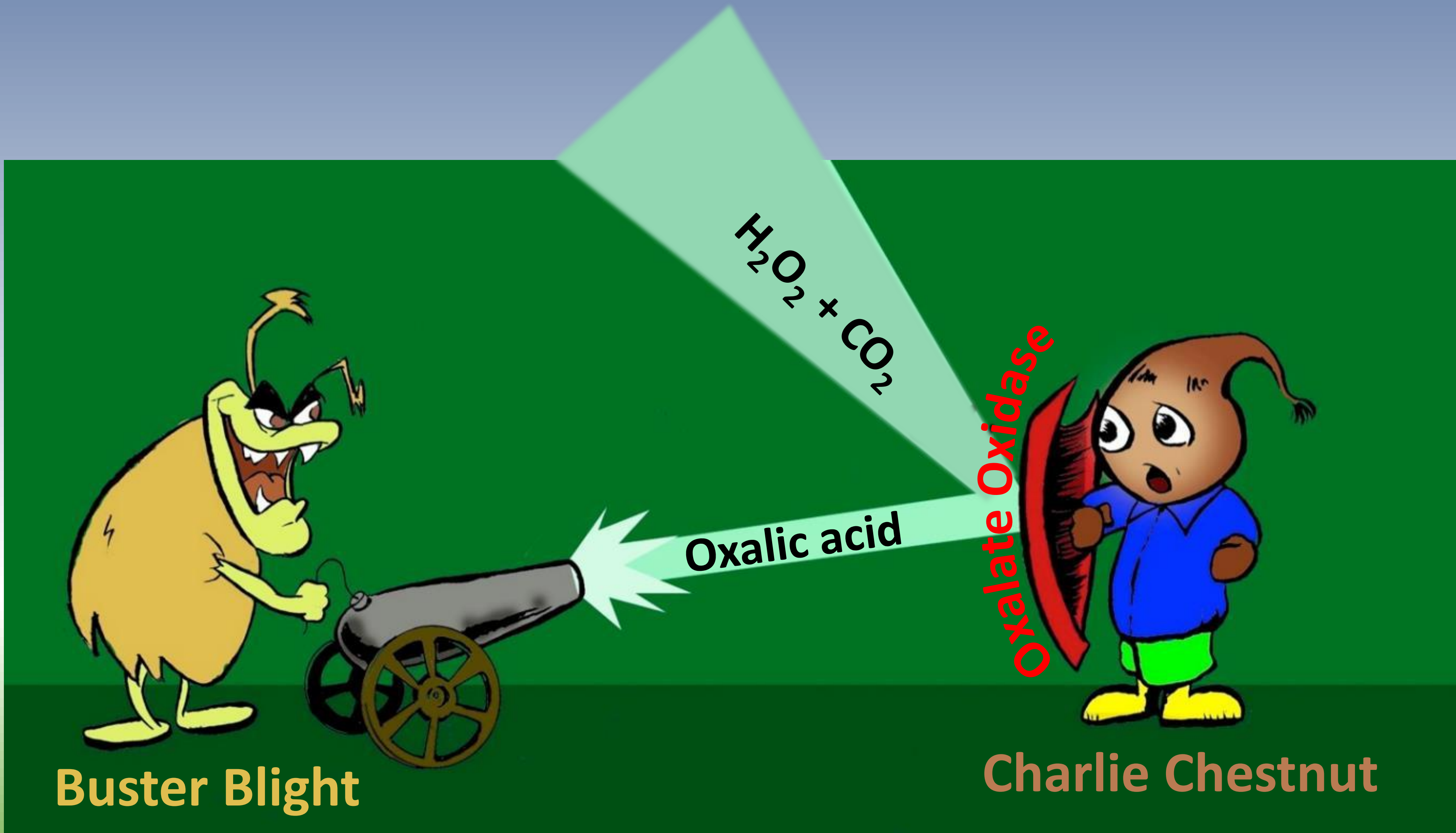
Banana

Strawberry

Cocoa

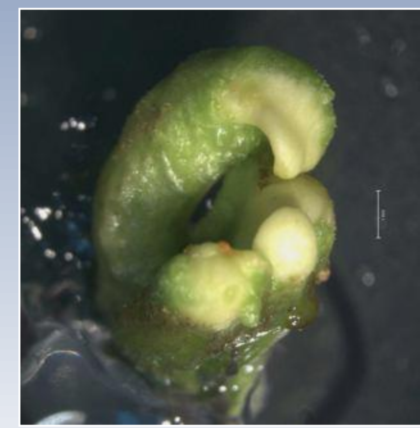
Many others

Alternative to chemical equation…

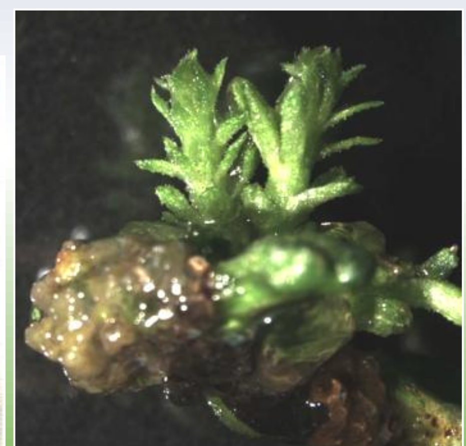


Isolation, Transformation, Propagation

New Gene/Trait, via *Agrobacterium*



(Many years of research…)



Crossing transgenic with non-transgenic American chestnut trees

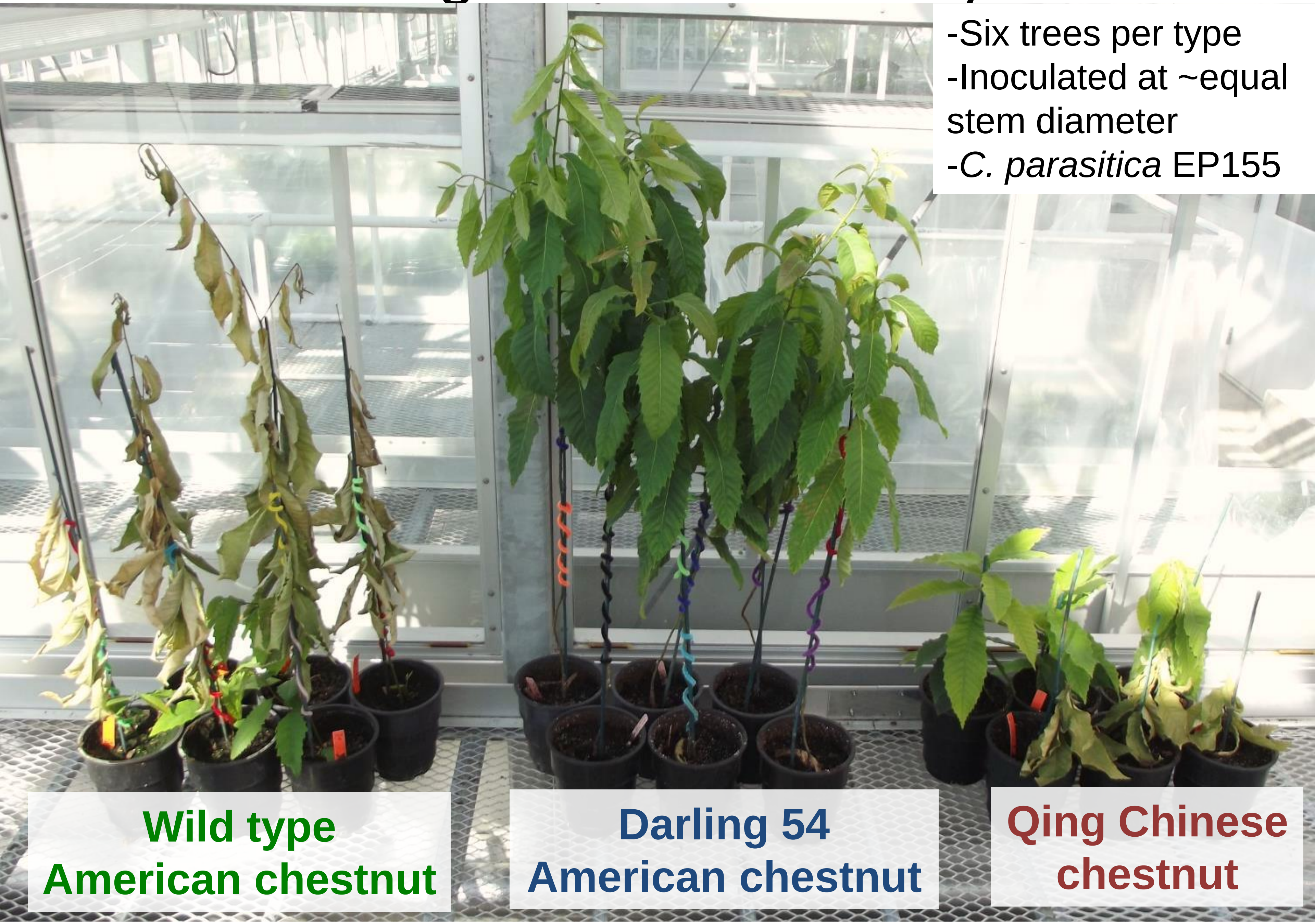
High-light production of pollen in less than one year

Tissue culture
vs
Seedling production

-Genetic diversity
-Ease of distribution

'Darling11' T1 (F1) seedling

Small Stem Blight Resistance Assay – 6 weeks



Wild type American Chestnut

Transgenic American Chestnut

Tolerance vs. Resistance

Non-target Comparison Studies
(Other species controls for context)
Consistently: No enhanced risks *compared to trad. breeding*

- Mycorrhizal colonization of transgenic roots (GH, field)
- Tadpole growth & development with transgenic leaves
- Bee feeding/use of transgenic pollen
- Nutrition of transgenic nuts
- Caterpillar feeding on transgenic leaves (+ tri-trophic)
- Aquatic insect growth/survival on transgenic leaves
- Transgenic leaf decomposition rates
- Native seed germination through transgenic leaf litter
- Native plant abundance near transgenic trees
- Growth rates, form, etc.
- Metabolomics (similarity of small molecules)
- Transgenic inheritance from transgenic pollinations, survival/expression/growth/blight resistance of offspring

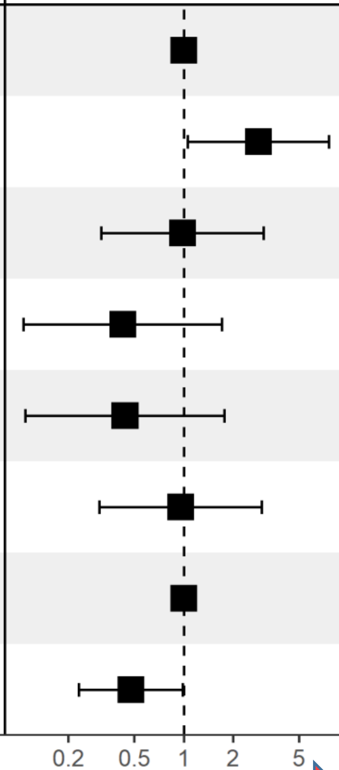


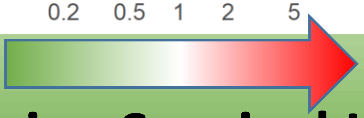
Tadpole Development and Survival in Deciduous Leaf Litter (Simulated vernal pools?)

- Vernal pool habitats & amphibians (thanks to herpetologist collaborators)
- Wood frog tadpoles in jars with leaves
- Six Leaf types:
  - Sugar Maple
  - American Beech
  - Chinese Chestnut
  - Hybrid (AC x CC) Chestnut
  - Non-transgenic American Chestnut
  - Transgenic (Darling 4) American Chestnut
  - No leaves (supplemental food only)
- With & without supplemental food

Tadpole Survival:
Cox Proportional Hazard Model

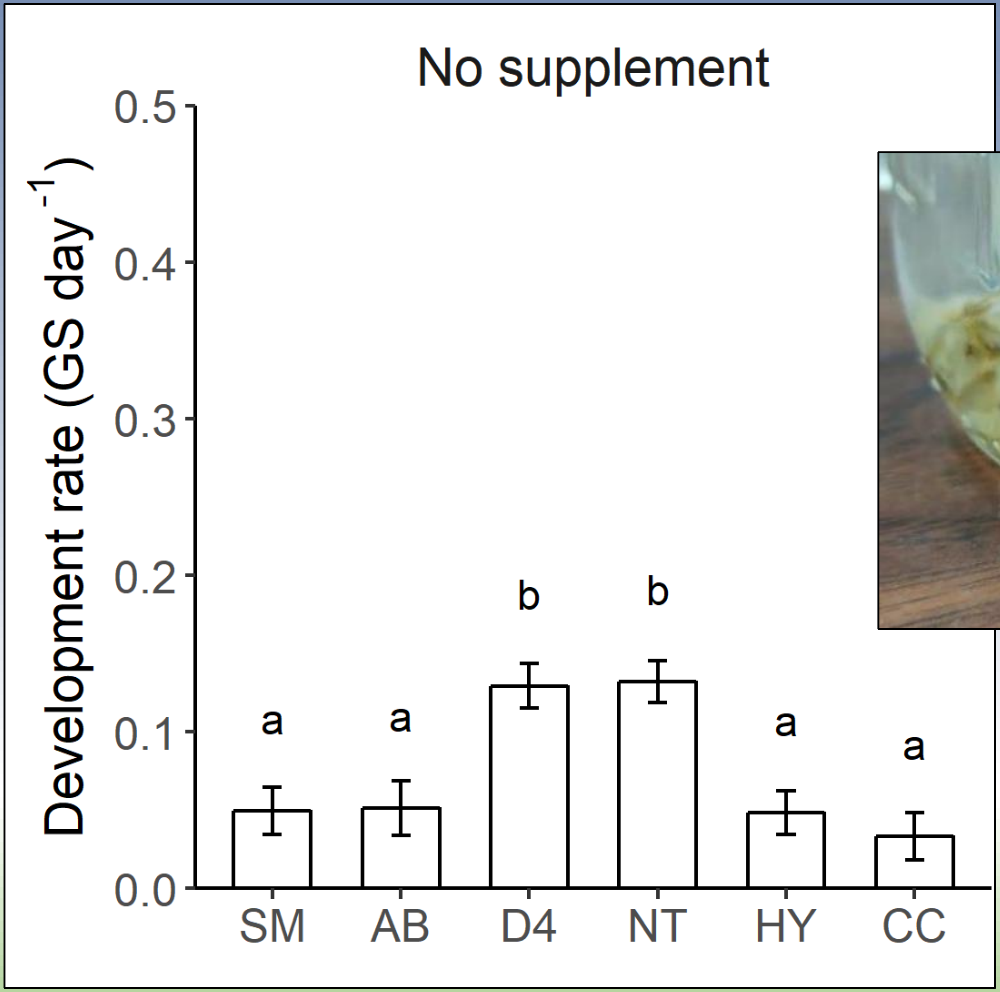
Variable		N	Hazard ratio		p
Leaves	SM	30		Reference	
	AB	30		2.84 (1.06, 7.60)	0.04
	D4	30		0.99 (0.32, 3.06)	0.98
	NT	30		0.43 (0.11, 1.71)	0.23
	HY	30		0.44 (0.11, 1.76)	0.25
	CC	29		0.96 (0.31, 2.97)	0.94
Supplement	No	89		Reference	
	Yes	90		0.48 (0.23, 0.98)	0.05



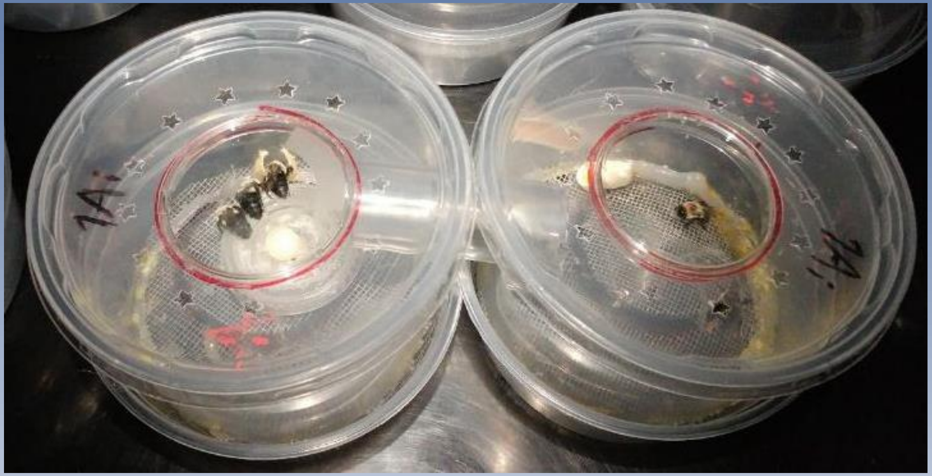


Increasing Survival Hazard

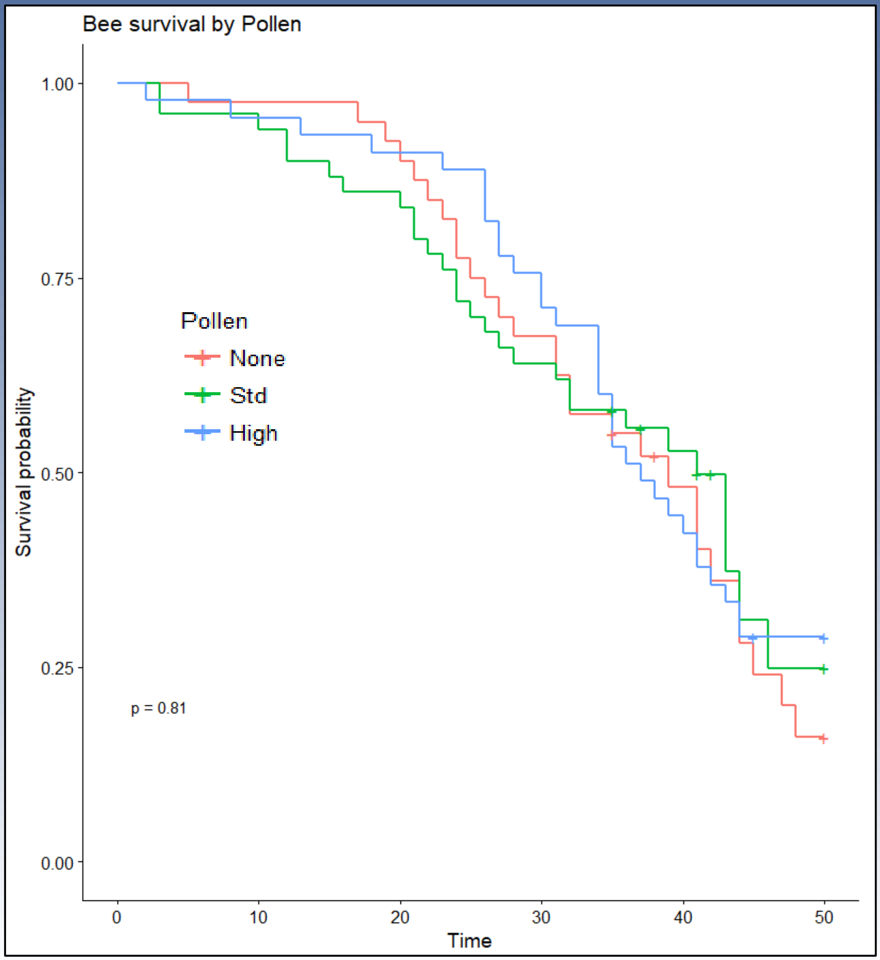
Tadpole Development

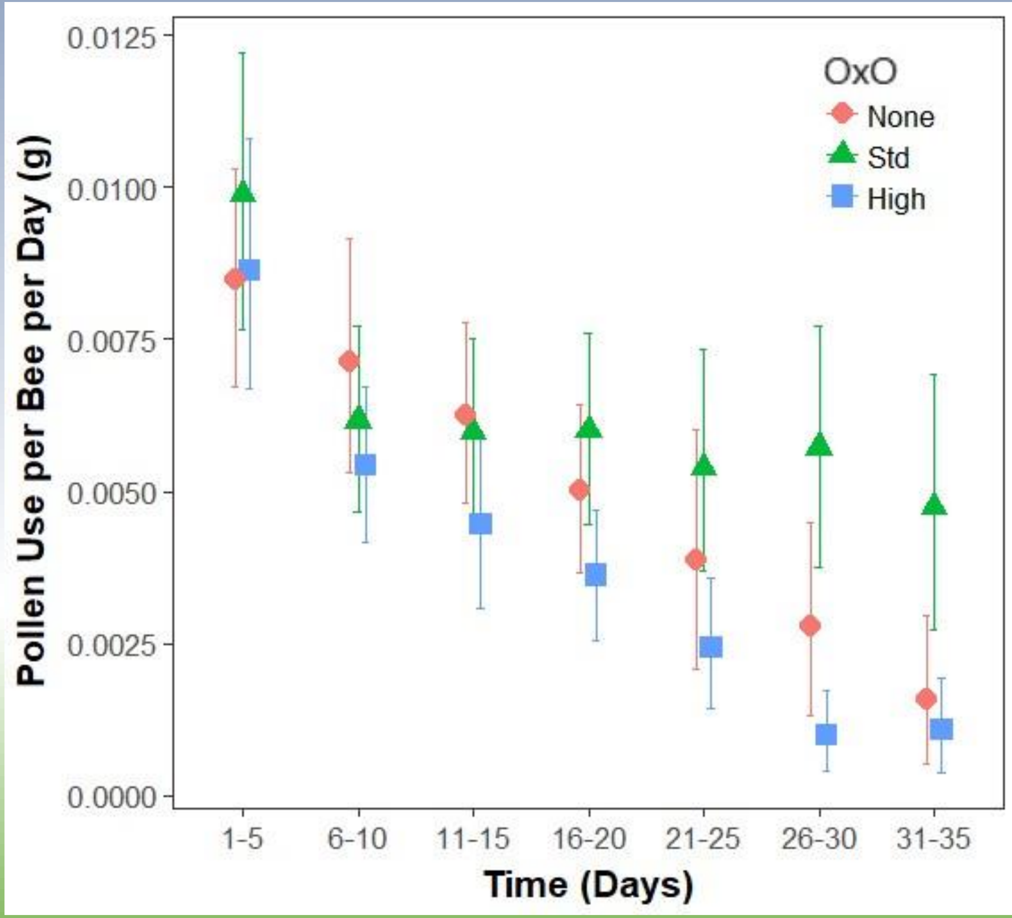


Bumble Bees and Chestnut Pollen



- Microcolonies
- Added 2 concentrations of OxO enzyme to pollen
- Tracked survival, pollen use, hive construction, reproduction

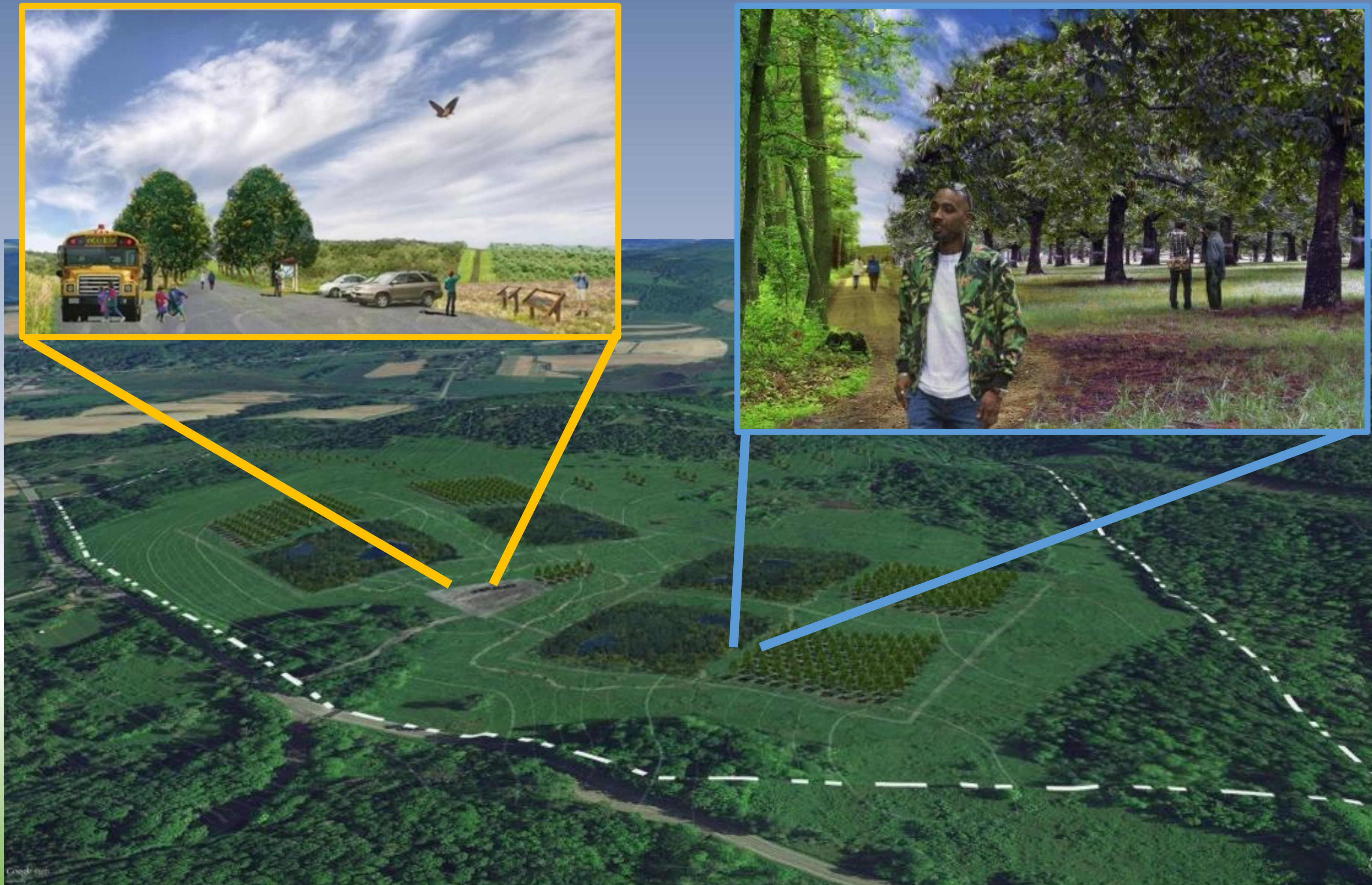

Bee survival by Pollen
Survival probability
1.00
0.75
0.50
0.25
0.00
Pollen
None
Std
High
p = 0.81
0
10
20
30
40
50
Time


Pollen Use per Bee per Day (g)
0.0125
0.0100
0.0075
0.0050
0.0025
0.0000
OxO
None
Std
High
1-5
6-10
11-15
16-20
21-25
26-30
31-35
Time (Days)

Nutrition
(+ Lack of allergens & toxins)

Data Source:	2017 Medallion Analysis		2016 Medallion Analysis						Database / Labels
Chestnut Type:	American x Darling58 T1	American x Darling58 NT	American (McCabe) x Darling4 T1	American (McCabe) x Darling4 NT	American (McCabe) x B3F3	American (Wisconsin) x OP	American (Moss Lake) x OP	American (Zoar) x OP	Chinese
Serving Size:	50g (~15 nuts)	50g (~15 nuts)	50g (~15 nuts)	50g (~15 nuts)	50g (~15 nuts)	50g (~15 nuts)	50g (~15 nuts)	50g (~15 nuts)	50g (~5 nuts)
Calories	100	100	120	120	120	120	80	110	110
Cal from fat	20	20	40	40	40	30	10	20	10
Total Fat (g)	2.5	2.5	4.5	4.5	5.0	3.5	1.5	2.0	1.5
Sat. Fat (g)	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5	0
Trans Fat (g)	0	0	0	0	0	0	0	0	0
Polyunsat. fat (g)	0.5	0.5	1	1	1	0.5	0.5	0.5	0
Monounsat. fat (g)	1.5	1.5	3	3	3	2	0.5	1.5	0.5
Sodium (mg)	0	0	0	0	0	0	0	0	0
Total Carb. (g)	18	18	19	19	18	19	17	20	25
Fiber (g)	3	3	5	5	5	5	3	5	3
Protein (g)	1.5	1.5	1.5	1.5	1.5	2.0	1.5	2.5	2.0
Vitamin A (% DV)	not tested	not tested	0	0	0	0	0	0	2
Vitamin C (% DV)	0	0	25	15	10	10	15	20	30
Calcium (% DV)	2	2	1	1	1	3	2	3	1
Iron (% DV)	0	0	4	4	4	4	3	4	4

Long-term Ecological Research (Pending regulatory approval)



Current Plantings: *Permit only*

- **ALL** outdoor plantings, pollinations, & interstate movements currently under USDA-APHIS-BRS permits
- No accidental release
- Requirements
  - Paperwork! Mapping, observations, reporting, etc.
  - Site inspections
- Pollination/flowering depends on site and containment methods
  - Essentially have to bag or remove all flowers

Federal Regulatory Agencies

- Canada

- FDA (Safety of food & feed)
  - Nutrition, composition, allergens, toxins
  - Technically voluntary
  - Response: no further questions

- EPA (Safety of pesticides)
  - "Mitigation" wording
  - Tolerance limit / exemption
  - Registration (renewed or replaced, never "de-regulated")

> under the Act (7 U.S.C. 136a(a)). FIFRA section 2(u) defines "pesticide" as: "(1) Any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest, (2) any

U.S. Regulatory Agencies – cont'd

- USDA-APHIS (safety for agriculture & environment)

When a developer has collected enough evidence that a GE organism poses no more of a plant pest risk than an equivalent non-GE organism, the developer may petition APHIS to determine non-regulated status for the GE organism. If the petition is approved by APHIS, the GE organism may then be introduced into the United States without any further APHIS regulatory oversight.

- Plant Pest Risk Assessment (potential new risks of modified product, potential weediness)
- National Environmental Protection Act (Environmental Impact Statement)
- Open comment periods
- Notifications to indigenous groups, other stakeholders

Non-Regulatory Groups

- Some US federal agencies have “consulting” roles
  - USFS (USDA)
  - NPS, FWS (Dept. of Interior)
- High-profile environmental groups
  - Not regulatory, but opinions may matter to regulators
- General public, via open comment periods
- Representatives
- TACF
- Other?

Regulatory Considerations

- Timeline: 2-5 years (?) after submission
- Lots of writing/preparation; no previous template
- Covers all offspring from "de-regulated" parent
- Privacy & security vs. transparency
- Community involvement and support is key
- Patience! (Tree research & fed. gov't regulations…)

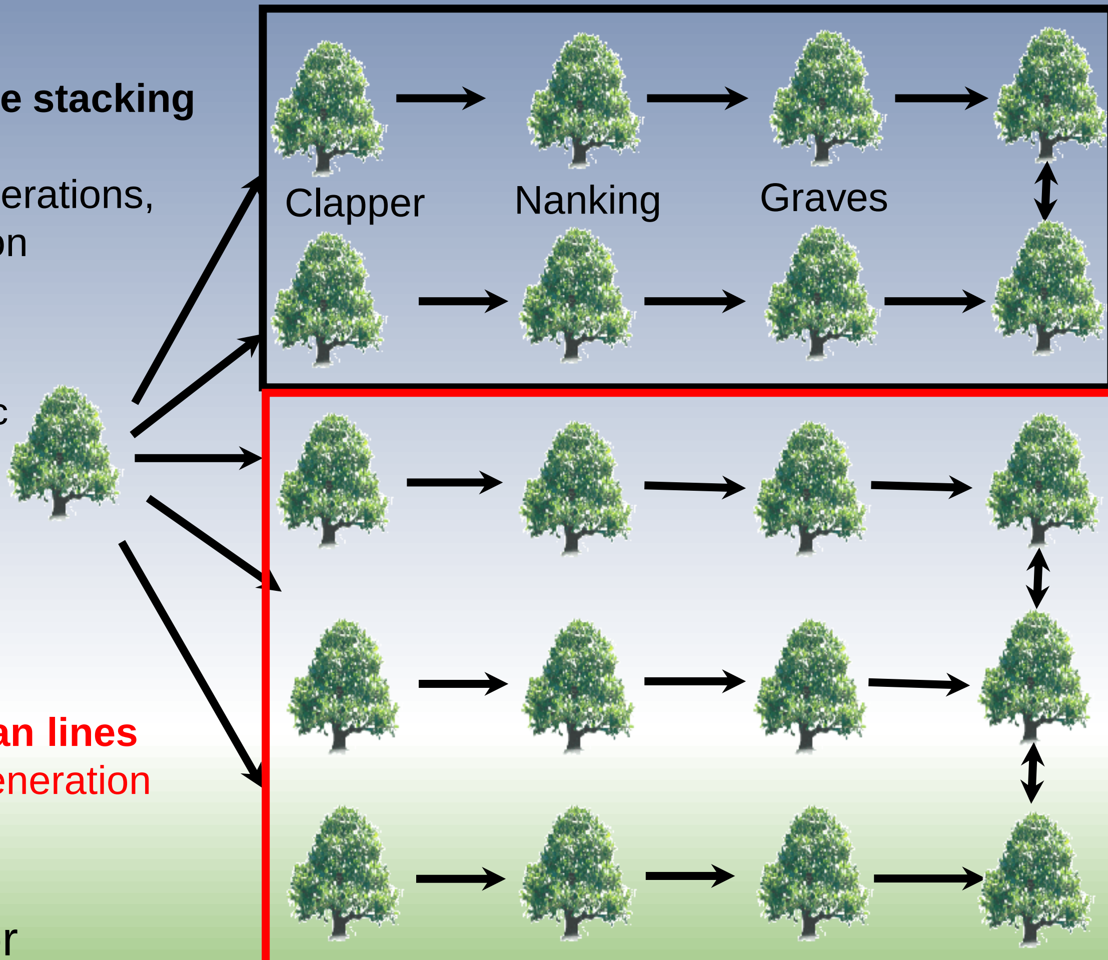
Potential restoration plans, outcrossing for genetic diversity (from TACF)

THE AMERICAN CHESTNUT FOUNDATION®

3 generations of outcrossing to dilute the founder genome

Backcross + OxO resistance stacking
Outcross to 200 backcross selections/generation x 3 generations, 1 resistance source/generation

Transgenic American chestnut



Create diverse pure American lines
Outcross to 300 Americans/generation x 3 generations

Pollen, female flowers, or seed from rare wild trees

Intercross to generate large quantities of seed for restoration

x 500 American chestnuts per generation

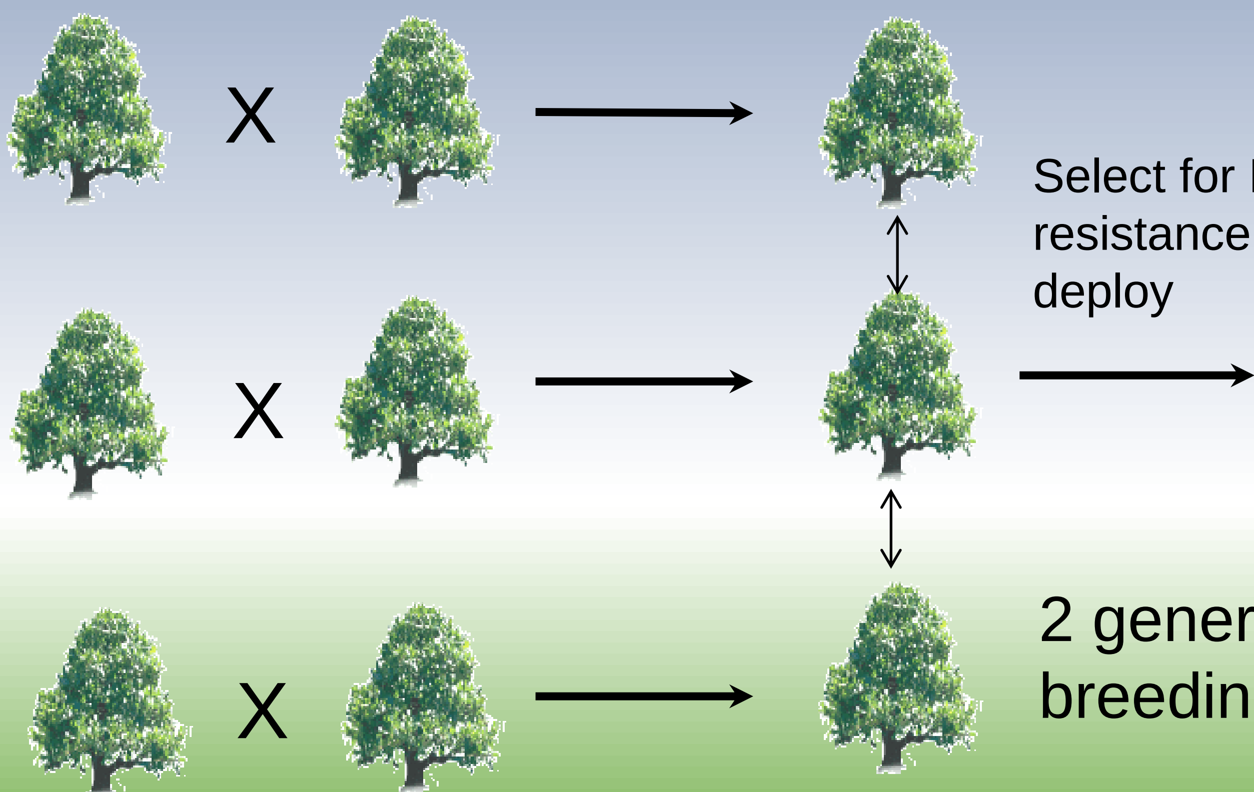
Additional breeding required to combine resistance to both blight and Phytophthora Root Rot

THE AMERICAN CHESTNUT FOUNDATION®

PRR resistant BC3-F2 selections

Blight resistant transgenic outcross progeny

Intercross to increase PRR resistance



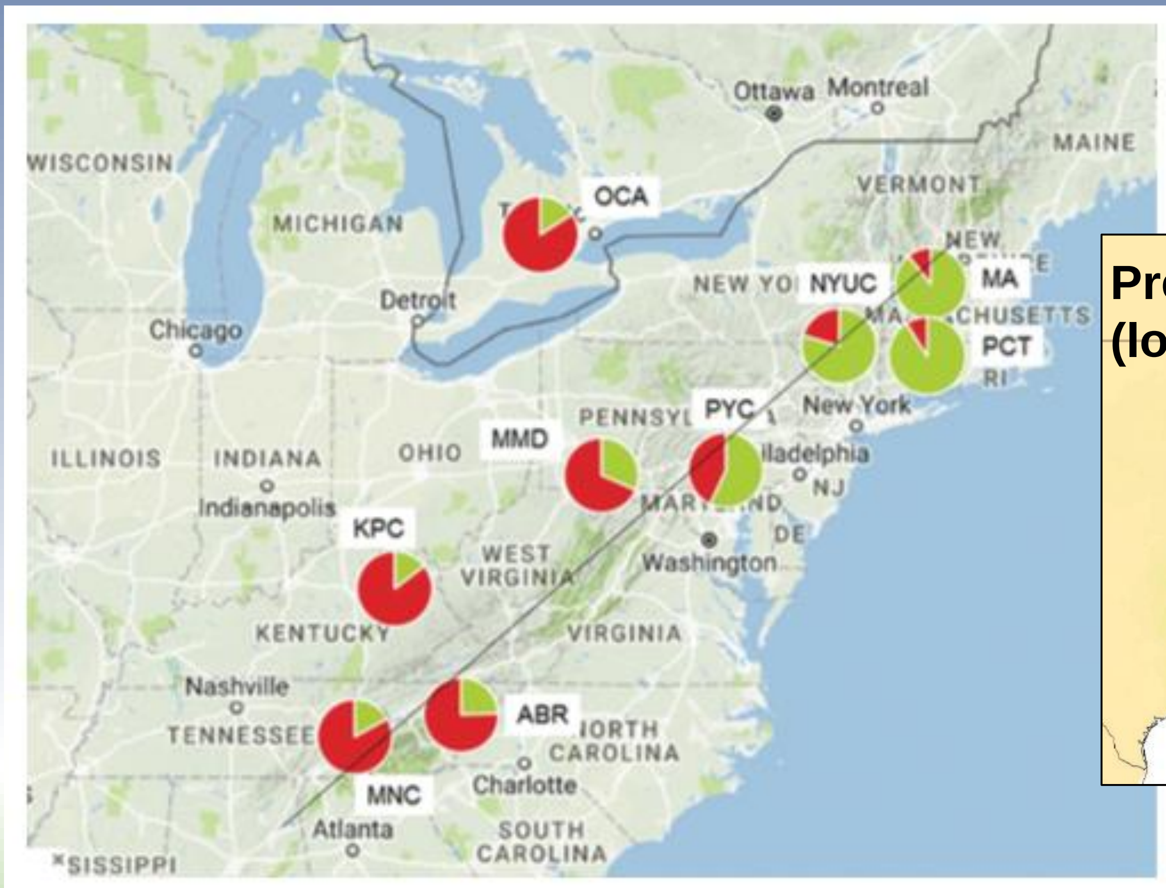
Select for PRR resistance and deploy

2 generations of breeding and selection

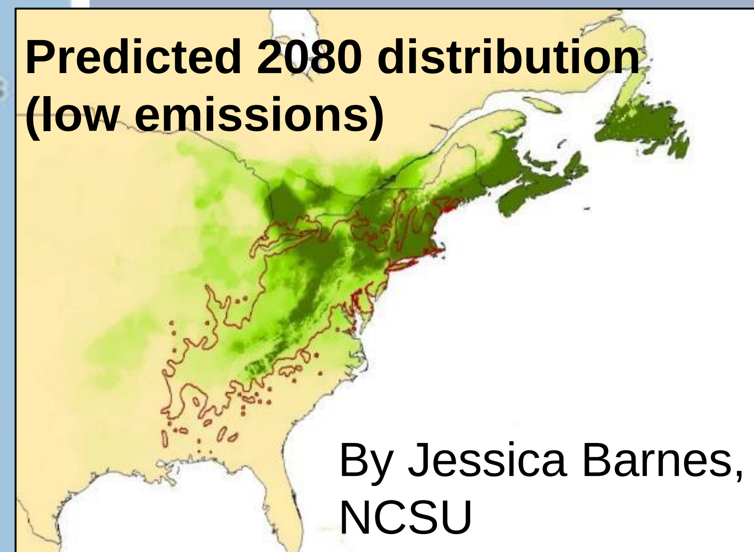
Responses so far

- Regulators are encouraging and helpful; laws are tricky
- Public surveys
- TACF
  - NY Chapter, national
  - Unique opportunity
- Public interactions
  - Minimal opposition (so far)
  - Happily discuss legitimate concerns
  - Some "anti-" arguments are conflated or ill-informed
  - Vast majority of articles and presentation responses are thoroughly positive
  - Many requests for trees!

American Chestnut Diversity, Future Restoration Considerations



Assisted migration?



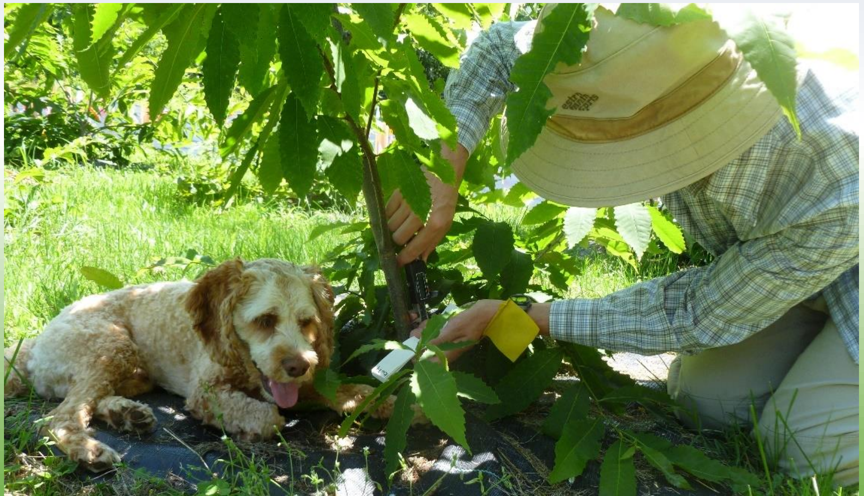
Red and green represent weakly differentiated subpopulations of American chestnut. Red subpopulation more genetically diverse than green

Gailing & Nelson (2017) *Botany* v95:799-807

Thoughts from this scientist:
Roles, responsibilities, obligations?

(Biotech for restoration)

- Who initiates research? Benefits? Profits?
- Who manages restoration?
- Restoration isolated from R&D?
- Who is involved in making decisions? (Workshop!)
- Who communicates with public? How?
  - Regulators, scientists, restoration group
  - Consultation? Who?



Bigger picture

- Chestnut firsts: forest-type tree, non-profit, restoration
- Discuss and evaluate (regulate?) in context of alternatives
- Other forest tree research in process
  - Elm, ash, walnut, western pines
  - Some planting permits, no regulatory submissions (yet)
- Responsible use
- Chestnut as a **success story**
  - Ohi’a, Coral

Thank you!

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www.esf.edu/chestnut