FALL 2020 ~ ISSUE 3 VOL. 34

Chestnut

THE JOURNAL OF THE AMERICAN CHESTNUT FOUNDATION

A BENEFIT TO MEMBERS

THE AMERICAN CHESTNUT FOUNDATION

Biotechnology ~ COVID Field Work ~ Teaching Hope ~ Search for American Chestnut ~ Using Transgenic Pollen

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Lisa Thomson

President and CEO

DEAR CHESTNUT FRIENDS,

2020 will be remembered like no other year in our lifetimes. While COVID-19, political, and social unrest has dominated the news, we all crave constants in our daily routines and connections with people and causes important to us.

Keeping our hearts and minds focused on positive goals and embracing optimism will sustain us. This is why so many of you in our greater TACF community have been a tremendous source of comfort and encouragement this year, along with surprising generosity.

When the pandemic hit us all late March, I was understandably concerned about the resilience and sustainability of our relatively small non-profit organization. Could we move our mission forward with restricted travel, work from home scenarios, and limited physical contact with each other? Would the farm and field staff be able to maintain our prized orchards while staying safe? The answer, so far, has been a resounding "yes!" Although no substitute for our festive in-person gatherings, we have embraced virtual technology to meet, share ideas, and even grow our membership.

Between January and the end of August, we gained 736 new members and are tracking remarkably steady donation support comparable to past years. Two private foundations even gave extra grants beyond their past annual giving. This extraordinary show of loyalty, for an organization that receives 94% of its revenue from private philanthropy, is the reassurance we need to stay laser focused on mission success. We are deeply grateful to all of you for your continued dedication.

The chance to save a species this impactful and symbolic is worth doing, even during the most challenging of times. To give you some perspective, the effort to rescue the American chestnut from extinction began soon after the blight's discovery in 1904. After more than a century of scientific study and experiments, through two World Wars, a Great Depression and Recession, and other unprecedented global events, our spirits have not dampened. We will prevail, and healthier forests and human communities will be our rich reward.

With much gratitude and great hope for your well-being,

Lisa Thomson, President and CEO
The American Chestnut Foundation

Charity Navigator is America's largest and most influential charity rater. The American Chestnut Foundation received another 4-star rating in 2020, making this the seventh year in a row we have earned the organization's highest ranking.

Chestnut Cross Section

Foresters call cross sections of wood "cookies" because of their shape. Each ring indicates the annual growth of the tree. The wider the ring, the better the growing conditions were that year. This cookie illustrates the rapid growth rate of American chestnut. It is one of several cross sections displayed at our national office in Asheville, NC.

THE AMERICAN CHESTNUT FOUNDATION™

WHAT WE DO

The mission of The American Chestnut Foundation is to return the iconic American chestnut to its native range.

CONTACT US

chestnut@acf.org
acf.org
facebook.com/americanchestnut
twitter.com/chestnut1904
instagram/tacf1983

TACF National Office
50 N. Merrimon Avenue
Suite 115
Asheville, NC 28804
(828) 281-0047

Meadowview Research Farms
29010 Hawthorne Drive
Meadowview, VA 24361-3349
(276) 944-4631

Mid-Atlantic Regional Office
The American Chestnut Foundation
900 Natural Resources Drive
Charlottesville, VA 22903
(828) 281-0047

New England Regional Office
UVM Aiken Forestry Service Labs / USFS NRS
705 Spear Street
South Burlington, VT 05403
(802) 999-8706

North Central Regional Office
Pennsylvania State University
206 Forest Resources Lab
University Park, PA 16802
(814) 863-7192

Southern Regional Office
50 N. Merrimon Avenue
Suite 115
Asheville, NC 28804
(434) 906-9312

Hannah Pilkey and Kaitlin Breda pollinating *C. dentata* mother trees with T_1 transgenic pollen to produce T_2 generation trees in SUNY-ESF orchards. Photo by William Powell.

Biotechnology

FOR BLIGHT RESISTANCE

By Erik Carlson, Research Project Assistant,
SUNY College of Environmental Science and Forestry

The restoration of the American chestnut is a task of great scale, both in size and complexity. The primary obstacle to restoration is chestnut blight, but other challenges face the species including Phytophthora root rot and chestnut gall wasp. To address these challenges, an integrated strategy for restoration is being deployed that combines the tools of breeding, biotechnology, and biocontrol: the 3BUR plan. This article focuses on biotechnology.

Why genetic engineering?

TACF's goal from the beginning has been to produce a tree as close to the original American chestnut as possible in order to be able to fulfill its previous niche as a forest canopy tree species. American chestnut has adaptations to the eastern North American forest ecosystem that are absent from Asian chestnut species, including its greater height which allows it to compete with other trees in a dense forest canopy. Backcross breeding achieves the goal of preserving American chestnut traits by repeated crossing of hybrid trees with American chestnut to dilute non-resistance genes from Chinese chestnut, resulting in B_3F_3 trees that inherited a majority of their genes from American chestnuts.

In contrast, genetic engineering introduces one or a few known genes, preserving the entire American chestnut genome. This eliminates the need to breed out unwanted genes, and thus reduces the chance of losing or diluting the tree's adaptation to its local environment. In subsequent outcrossing, selecting for a single added resistance gene is a much simpler process than selecting for multiple, independently inherited resistance genes. With a single dominant gene, half of all offspring inherit blight resistance, a much higher proportion than in backcross breeding. Trees containing the blight resistance gene are also capable of producing wild-type American chestnut seeds without

Chinese, OxO American, and wild-type American chestnut field inoculations with *Cryphonectria parasitica*

Chinese chestnut

OxO American chestnut
Sibling OxO+

Wild-type American chestnut
Sibling OxO-

the resistance gene, a unique ability not shared with hybrid breeding which may be useful for future conservation efforts.

What are the drawbacks of genetic engineering?

Adding blight resistance genes through genetic engineering requires the transformation of individual plant cells in tissue culture. Whole trees are generated from a single transformed cell, after selecting those cells from others that did not receive the gene. The first generation of trees generated from those cells are genetically identical. This is ideal for testing and assessing levels of blight resistance in comparison to clones that do not contain the gene, but you cannot rebuild a species with genetically identical trees. This is where one of the other complementary tools of 3BUR comes into play: breeding. Crossing pollen from blight-resistant trees with surviving American chestnut mother trees can integrate genetic diversity into the breeding pool of future restoration populations. As more generations of outcrosses are made, diversity continues to build in the blight-resistant American chestnut offspring (Westbrook et al. 2020). These trees can also be crossed with trees from the backcross breeding program to incorporate additional resistance genes from Chinese chestnut that may protect against both chestnut blight and Phytophthora root rot.

How do you choose a gene to add?

Before creating trees resistant to the chestnut blight, it was important to understand how the blight fungus attacks chestnut trees. Studies on hypovirulent strains of blight fungus used in biocontrol revealed one of the main weapons of the fungus: strains that produced high levels of oxalic acid were the most damaging to trees, and conversely, strains that produced low levels of oxalic acid did very little damage to trees (Chen et al. 2010; Havir and Anagnostakis 1983). With this knowledge it was possible to develop a strategy to counter the attack of the fungus.

Oxalic acid (aka oxalate) is a common toxin in nature and a variety of species of plants, fungi, and bacteria have developed mechanisms to counter it. Many wild and domesticated plants contain an enzyme called oxalate oxidase that detoxifies the acid by converting it into carbon dioxide and hydrogen peroxide, natural compounds that are used by plants. Chinese chestnut likely utilizes a multiple enzyme pathway to break down oxalic acid, ultimately achieving a similar effect as oxalate oxidase.

The oxalate oxidase gene from wheat was added to American chestnut to detoxify the oxalic acid produced by blight (Zhang et al. 2013; Newhouse et al. 2014). The wheat gene was selected because it had been well studied, it is commonly eaten by people and animals, and it confers high levels of blight resistance in American chestnut. Although oxalate oxidase appears to accomplish its purpose in transgenic American chestnuts, new innovations continue to be sought: stacking multiple blight resistance


ESF students planting out T_2 transgenic seedlings in newly expanded diversity field plots representing 85 T_1 pollen/mother tree combinations. Photo by William Powell.

genes through either breeding or genetic engineering or gene editing are being investigated, and genes for resistance to Phytophthora root rot are also being explored.

Why can't I get one of these trees yet?

Genetically engineered trees are highly regulated by government agencies (USDA, EPA and FDA). This process helps to ensure that these trees are safe as a food source, and do not present an enhanced risk to the environment as compared to other restoration strategies, such as traditional breeding. However, it does mean that years of study and a lengthy application process are required before they can be released to the public. Many studies investigating the safety of the trees were completed and are currently under review (see "Safety Tests" three-part series in the 2020 winter, spring, and this issue of *Chestnut*), and even more tests are underway. ESF's "Petition for Determination of Non-regulated Status" has been accepted by the USDA and posted on the Federal Register, and is open for public comment through October 19, 2020. TACF is supporting this process by notifying its members and other stakeholders of the opportunity to submit positive comments. Personal stories and scientific backing for the use of biotechnology in conservation would be a great way to show your support for TACF's 3BUR efforts!

Options for submitting a public comment to the USDA:

- Visit TACF's website at acf.org and click the PUBLIC COMMENT PERIOD link on the homepage to access helpful resources.
- Go directly to the comment submission page on the Federal Register's website: http://bit.ly/fedreg-darling58
- Mail your comment to:
  Docket No. APHIS-2020-0030
  Regulatory Analysis and Development
  PPD, APHIS, Station 3A-03.8
  4700 River Road, Unit 118
  Riverdale, MD 20737- 1238

ESF State University of New York College of Environmental Science and Forestry

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Field Work

IN A TIME OF CORONAVIRUS

By Kendra Collins, New England Regional Science Coordinator, with contributions from chapter leaders throughout TACF's four regions

On the American chestnut calendar, March signals the arrival of spring and the onset of another growing season. We start to plan spring plantings and orchard clean-up, pot seedlings for future projects, and prepare for another field season working for the species we hold dear. But this past March brought along a new challenge – the COVID-19 pandemic. While eager to get back in the field, everyone's focus shifted to safety and limiting personal interactions. Stay-at-home orders were issued, TACF's spring board meeting was held virtually, staff offices closed, and our active chapter members were advised to cease all in-person work. But as the spring wore on and states began to relax some of

TACF's Dan Mckinnon and Jim Tolton burn rogued trees at Meadowview Research Farm's Duncan farm, while practicing physical distancing. Dan's TACF bandana keeps the cold air and ash out of his face, and doubles as a pandemic-safe face covering. Photo by Dan Mckinnon.

their restrictions, many began wondering about safe ways to continue our field work. Surely we couldn't all take a season off from our efforts to save the American chestnut! There was important work to do to keep our mission on track.

With an eye on continuing mission-critical tasks, TACF staff researched field work protocols from other agencies and organizations doing similar conservation work and developed a set of guidelines for our volunteers to follow when heading back into the field. Anyone at high risk or uncomfortable in any way was encouraged to stay home and safe. But for those chomping at the bit to get out, recommended preparations included a self-health check for symptoms, following any state guidelines on gatherings or travel, ensuring the site owner was comfortable with visitors, and assessing the need for the work under consideration. Anything that could be put off until a later date or season could wait. In addition, solo work, or work with household was encouraged. Guidance for small group activities with proper personal protection equipment (PPE) and physical distancing were implemented.

While field work required more planning and a major shift in methods, by following these guidelines field staff and TACF volunteers were able to accomplish meaningful work during what could have been a lost field season. In true TACF volunteer fashion, all chapters adapted their methods as needed to take care of critical tasks. Below are some highlights from chapters across all four of our regions, as well as a look at how TACF's field staff adapted to field work during a pandemic.

CT-TACF Chapter update from Jack Swatt

After a successful chestnut harvest in the fall of 2019, the CT-TACF Chapter was looking forward to spring planting of our first germplasm conservation orchards (GCOs) and establishing a third seed orchard in Connecticut. Because of the COVID-19 crisis, however, we had to adjust our process to keep everyone safe. All new GCOs were being hosted by private landowners who had already agreed to take on much of the work. I developed planting demonstration videos and several of the landowners, working solo, completed the planting and fencing process. For those needing assistance, one or two volunteers would schedule a visit and work alone.

Planting the seed orchard brought different challenges. Some preparation was done individually, but we needed more volunteer help to get all the nuts planted at the appropriate time. Tasks were divvied up and the landscape fabric

Volunteers with the Winchester Land Trust and CT-TACF Chapter successfully installed a new seed orchard, while following strict physical distancing from those not living in the same household. Photo by Jack Swatt.

was marked at 1′ intervals, which made maintaining proper distance easy. The project took a bit longer because of safety precautions, but everyone seemed to enjoy outdoor time and an opportunity to participate in the orchard planting. The ability to make adjustments and work independently carried us successfully through our busy planting season in the year of the pandemic.

PA/NJ-TACF Chapter update from Louise Aucott

Five PA/NJ-TACF Chapter volunteers met Friday, June 5 in the F_1 and F_2 orchards at Codorus State Park in Hanover, PA. Leaf samples were harvested for genotyping from the roughly 300+ F_1 and F_2 survivors, part of the Pennsylvania Chestnut Timber Tree Program (originally the F123 Program) initiated by chapter members Bob Leffel and the late Dave Armstrong. Kelly Ford, a Cordorus Environmental Interpretive Technician, joined chapter members Jay and Marguerite Brenneman, Louise and Mike Aucott, and Betsy Murtha. The group cooperated to avoid the pitfalls of community transmission of COVID-19. We came armed with masks, antibacterial wipes, hand sanitizer, and most importantly, a shared understanding of social distancing. Dividing the work logistically, we were able to sample the F_1 and F_2 orchards in about six hours. One person loaded collection bags with pre-printed tree ID labels and desiccant packs, labeling each on the outside, and then handing them off in batches to other team members to distribute to the base of each tree. The leaf gatherers followed, picking and wielding pole-mounted clippers as needed, masking only when assisting one another at close range. Our risk-reduction measures: stay outside; share no food, drink, hugs or handshakes; and skip group photos. Success!

KY-TACF Chapter update from Ken Darnell

On Saturday, June 6 Jennifer Koslow, associate professor at Eastern Kentucky University (EKU); KY-TACF board members; and a group of students, teachers, and volunteers, converged near EKU's Regional Seed Orchard in Richmond KY. Their goal for the day? To inoculate 1,000+ three-year-old trees.

However, this year was like no other when it came time to perform the work. Before setting out, Jennifer coordinated the safety protocol steps with Tom Saielli, TACF's regional science coordinator for the mid-Atlantic and southern regions. The two collaborated, making sure the work would be accomplished safely. Participants, guided by Jennifer, were instructed to drive separately to the orchard, engage in social distancing, and wear masks if working within 10' of another volunteer. Prolific use of hand sanitizer and disinfectants for gear was also a requirement.

Jennifer then coordinated an assembly line with each volunteer completing a step: removal of tree shelters, marking and sterilizing, drilling the

Bags at base of trees and leaf sampling team at work. L to R: Betsy Murtha, Jay Brenneman, Marguerite Brenneman. Photo by Mike Aucott.

Volunteers, students, and staff at EKU discuss safety instructions prior to inoculating the EKU seed orchard. Photo by Ken Darnell.


GA-TACF Berry College interns, Noah Howie and Marshall Lynch, inoculate seedlings at a chestnut backcross orchard with face masks on while maintaining a safe distance. Photo by Martin Cipollini.


Riona Collins, junior field technician to mom Kendra Collins, helped assess flower development and pollination timing of a germplasm conservation orchard in Burlington, VT. Photo by Kendra Collins.

hole, and applying inoculum and tape. Everyone paced themselves to balance the work load and keep safe spacing. It is always a joy when "chestnut veterans" have the opportunity to work with motivated students interested in biology, botany, ecology, horticulture, and wildlife. Our future is in good hands!

GA-TACF Chapter update from Martin Cipollini

The introduction of a pandemic also introduced new and unique field work challenges, but the GA-TACF Chapter pressed on throughout the spring and summer, thanks to a number of dedicated volunteer stewards who respectfully followed TACF's safety protocols.

When field work could be organized safely, outdoors, and with minimal numbers, face masks and social distancing became the norm. In mid-June, for example, Berry College interns Noah Howie and Marshall Lynch assisted Martin Cipollini, professor of biology at Berry College and GA-TACF science coordinator, in conducting a field assay to assess blight resistance in relatively young trees. Similar to other chapters, minimal pollinations were conducted with fewer volunteers who wore face masks and practiced social distance guidelines. Despite COVID-19, good and productive work is being accomplished in the chapter.

TACF Field Staff

TACF's Regional Science Coordinators (RSCs) and the staff at the Meadowview Research Farms made significant changes in the early stages of the pandemic. Field work plans were assessed for safety and feasibility, travel was greatly limited, and essential work was prioritized. The RSC's remote offices all closed and access continues to remain restricted, leaving them largely working from home into the foreseeable future. Meadowview staff stayed home for office work (and enjoyed time with their furry friends), and developed a system for limiting the number of people on site at any one time. They used a group text to ensure everyone was safe and accounted for when working primarily solo. In this way, the farm was fortunately able to carry on with most planned work for the season.

In addition to closed offices, the RSCs all have small children and the closure of daycares and schools made for busy homes, hectic schedules for two working parents, and rewarding, but challenging days. Tiny faces and voices made appearances at online staff meetings, and moving between data analysis and Duplo block creations became a new required skill. With field travel restricted, volunteers and interns were also relied on to do more with only virtual or remote supervision. While spring usually brings the resumption of extensive field travel for the RSCs, the pivot to staying put was an adjustment! Limited travel was resumed for mission-critical field work during inoculation and pollination season, but it wasn't until mid-summer that anyone on staff travelled beyond home state borders.

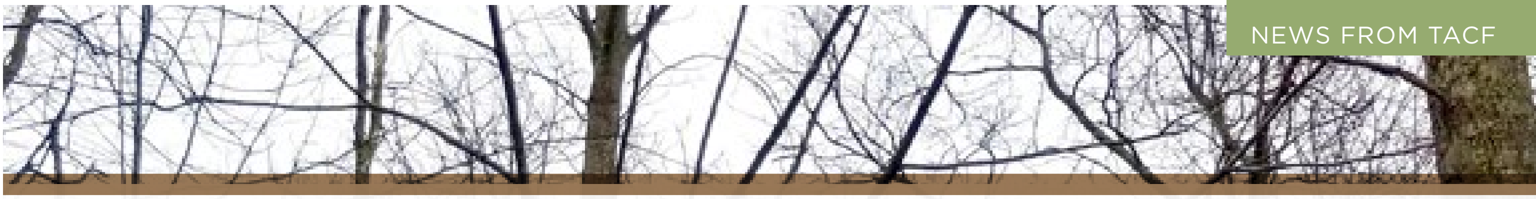
TACF'S LIVE CHESTNUT CHAT SERIES

A New Way to Engage

In the early stages of the COVID-19 pandemic, when stay at home measures began taking shape, staff at The American Chestnut Foundation (TACF) immediately began brainstorming new ways to engage supporters during these unprecedented times. The result was a live online series we call Chestnut Chat. Occurring every other Friday at 11:30AM, these lively Chats offer informative presentations and helpful advice about any and all matters related to American chestnut restoration. Topics cover everything from drones, to tips on identifying the tree in forests, to the latest research developments. An interactive Q&A session is offered during each Chat, allowing ample time for participants to ask questions.

The series has been a huge success and TACF plans to continue offering it into the foreseeable future. Information about upcoming Chats and how to join can be found on our website calendar at acf.org/events/category/tacf/ or by reading the electronic email invitation sent each week a Chat is scheduled to take place. If you are not always able to participate in the live sessions, or are interested in watching past episodes, each is recorded and published on our website's Chestnut Chat Series page at acf.org/resources/chestnut-chat-series/.

Interested in a particular topic yet to be covered?
Email TACF Director of Restoration Sara Fitzsimmons (sara.fitzsimmons@acf.org) to share your idea and why the issue is important to you.



Documenting Large Surviving American Chestnut Trees

IN EASTERN WEST VIRGINIA

By Darrell Dean, Jr. and Robert Sypolt, WV-TACF Chapter

Darwin Bergdoll leans on Tree #5, which displays beautiful timber form. Jim Bowen stands to the right of the tree. Photo by Darrell Dean, Jr.

Nearly seven years ago the authors and classmates from Rowlesburg High School in Rowlesburg, WV, became interested in the restoration of the American chestnut. This narrative is about our tours in Hardy and Pendleton counties in eastern West Virginia to document large surviving American chestnut trees.

In March, before COVID-19 regulations were implemented in the state, we traveled to Moorefield to meet with Jim Bowen, a service forester with the WV Division of Forestry, at his office in the WV Department of Agriculture Complex. Jim had agreed to show us some large American chestnut trees, one in Pendleton County and four in Hardy County; and the hunt commenced.

Leaving Moorefield, we traveled to a location southwest of Franklin to meet with Jean and Glenn Riggleman. They guided us through their property to an isolated American chestnut we identified as tree #1. This hearty specimen has a diameter at breast height (DBH) of more than 18 inches and is estimated to be over 50 feet tall. No blight cankers were visible and, in fact, the tree produced burs in 2019. Tree #1 has great timber form and is in the dominant crown class growing in an oak stand. Topographically, #1 is growing on a flat ridge toward a southeast aspect, at an elevation of about 3,055 feet.

After thanking the Rigglemans for access to their property and tree #1, the crew headed north to southern Hardy County, roughly 20 miles south of Moorefield. There, we connected with property owner Arlie Michael and forester Darwin Bergdoll, who works for Grant County Mulch, Inc. It should be noted that foresters working for wood product companies associate with many people, particularly loggers, in the wood product supply chain. Due to the wide area work distribution in a forest stand, loggers have more opportunities to encounter large surviving American chestnut than the casual chestnut hunter. Knowledge of these encounters is generally passed on to the company forester. Consequently, as a result of Darwin's work with loggers, he would lead us through Arlie's property

Jim Bowen brings down burs using a tomahawk. Photo by Darrell Dean, Jr.

Visible canker on tree #2. Photo by Darrell Dean, Jr.

onto a large private tract of land, leased by a hunting club, to four different chestnut trees.

Our first stop was tree #2. This tree has a DBH of 16.3 inches, is in a predominately oak stand, and its crown class is dominant. Recent bur production was absent as were other nearby American chestnut trees. Topographically, #2 is growing at the bottom of a slope with a southwest aspect, at an elevation of about 2,405 feet. This tree has relatively large cankers on the main trunk and appears to have a dead top. Last fall, in an effort to save it, Jim treated the tree with what he believes are hypovirulent strains of the chestnut blight fungus. Time will tell if the treatment is successful.

Jim also inoculated trees #3 and #4 with hypovirulent strains of the blight fungus. The loose and separated bark on tree #3 indicates blight fungus is present. (Notice in the photo the discontinuity in the bark above head-height and at the base of tree #3.) However, the tree has managed to persevere for many years. Tree #3 has a DBH of 18.4 inches, is growing in a predominately oak stand, in which it has a dominate crown. The tree produced burs in 2019. Topographically, its location is near the top of a ridge on a southeast-facing slope, at an elevation of about 2,800 feet. Tree #4, with a DBH of about 6 inches, is on the ridge above tree #3 and, sadly, appears to be in the late stages of chestnut blight. However, with hopeful optimism, Jim treated this tree as well. If tree #4 survives, it could be a potential source of pollen for tree #3.

Tree #5 is lucky to be alive! Not only has it successfully avoided death from the blight, it also escaped the "woodsman's ax." While harvesting the stand in which tree #5 grows, a logger recognized it as American chestnut and chose not to fell the tree. The logger then informed Darwin of its location and consequently, this tree, with very significant properties of blight resistance and timber form, was saved. Tree #5 has a DBH of 18 inches, a dominant crown in an oak stand, and was a prolific bur producer in 2019. The tree appears to be free of the blight fungus. It is one of the most magnificent American chestnuts we have observed. Topographically, it is growing at mid slope with a southwest aspect, and at elevation of about 2,495 feet. Notably, tree #5, with its superior resistance and timber form, is growing under the least favorable conditions in comparison to the others. That is, water storage of only 1 inch in the soil profile and soil productivity as measured by the oak site index (height of an oak tree at 50 years) is 60. The chestnut trees growing on the other sites had values as high as 72 inches and 85 respectively.

Our eastern West Virginia chestnut tour was a long but productive day. We savored our observations and reflected on the experience over an evening meal at O'Neils, a local restaurant in Moorefield, before returning to Preston County.

Tree #3 with bark discontinuity. L to R: Robert Sypolt, Jim Bowen, and Darrell Dean, Jr. Photo by Darwin Bergdoll.

Teaching Hope

WITH THE AMERICAN CHESTNUT

By Erin Hines, Environmental Educator

I still remember the first time I learned about the American chestnut tree. I was fifteen years old, on a grant-funded backpacking trip for high schoolers in the mountains of North Carolina. My trip leader was a student from a local college, and he seemed to know everything about every plant we encountered. One evening, he told us the story of the American chestnut, and I was wholly enthralled with the idea of massive trees with sweet nuts that I had only experienced in Christmas carols. He showed us small chestnut saplings sprouting from giant stumps that seemed half-rotted, half-petrified. I loved the delicate leaves with their sharp, even teeth. I was fascinated by the chestnut wood's ability to endure. And I was completely inspired by the idea of scientists working to bring this tree back through breeding and genetic research.

Erin (center, green shirt) in 2007 on Pinnacle Mountain, NC. The program, CLIMBE (Center for Learning and Investigation in Mountain Backcounty Ecosystems), aimed to educate high school students about climate awareness and water quality, and was sponsored by Montreat College.

So often we pose stories of past environmental disasters as cautionary fables, intending to lead our children down a different path than the one walked by previous generations. Sometimes we forget the old paths are established by years of travel, and divergence can seem impossible and overwhelming. The environmental issues of today are complex and systemic, and solving them can feel like setting off alone into thick wilderness. We lay this burden of trailblazing on the shoulders of our youth, with only our cautionary tales as guidance. Is it any wonder when they react with distanced interest, if interest at all, to our call to save the world? David Sobel, author of *Childhood in Nature*, calls this phenomenon "ecophobia." In his article, "Beyond Ecophobia," he asks, "What really happens when we lay the weight of the world's environmental problems on eight and nine year-olds already haunted with too many concerns and not enough contact with nature?"

The story of the American chestnut, and the work of The American Chestnut Foundation (TACF) to save

Erin holds burs from an American chestnut she found in 2015 on Table Rock Mountain in Pisgah National Forest, NC.

Erin stands by a European chestnut tree near Ponferrada, Spain, in August, 2018. The European chestnut has been an important industry and food source for the El Bierzo region of Spain for over 1,000 years.

the tree, is not a cautionary tale. It is a story of hope. As an environmental educator, I work with students aged kindergarten through twelfth grade, and I have incorporated the story of this magnificent tree in many lessons. When I tell this story, the students do not "check out." They understand they have a right to see this tree; they should know what this nut tastes like. They understand the importance of science to accomplish real progress, and see the effectiveness of long term vision and collaboration. They understand that individuals have the ability to make a difference through small adjustments over time.

There are many ecological concepts inherent in this story as well. Most recently, I created a video featuring the American chestnut (https://bit.ly/chestnut-biodiversity-video) as a way to explain the importance of biodiversity and involve students in the City Nature Challenge, a global bioblitz hosted annually in April. (Bioblitz, per National Geographic, "is an event that focuses on finding and identifying as many species as possible in a specific area over a short period of time.") Education this spring has been difficult, to say the least. Students in my county finished the semester virtually through self-paced lessons and assignments. Instead of visiting students in their schoolyard, I scraped together weekly videos demonstrating hands-on activities to get students away from their desks, balancing the inevitable screen time with time outside. In the midst of the COVID-19 crisis, when the physical and mental health of our students has been so critical, the American chestnut again gave me a story of agency and optimism.

While American chestnuts are no longer found knitted throughout our Appalachian canopy, students can still engage with this tree through historical photos of gigantic trunks and sightings of stubborn saplings sprouting in the understory. American chestnuts speak to what once was and could be again, with determination and persistence. It is an apt tale for the youth of today, as we equip them as best we can with the knowledge and skills to make environmentally responsible decisions. Like the American chestnut, they are our future, and our efforts will soon land in their hands. It is our responsibility to inspire them with their own capacity to contribute to a better world, and the restoration work of TACF provides just that sort of inspiration.

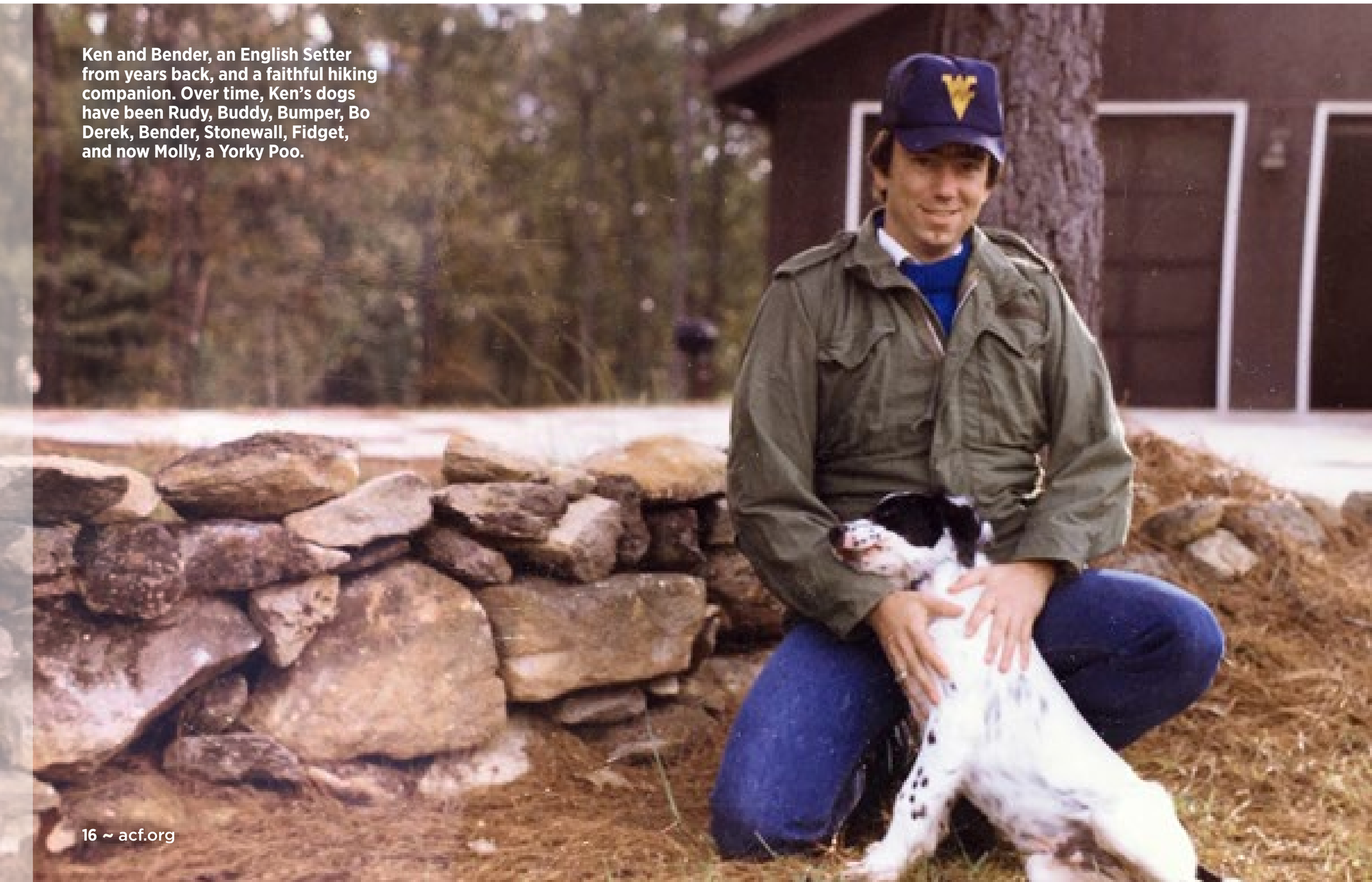
Erin Hines is an environmental educator for Gaston County's Soil and Water Conservation District in the Natural Resources Department, and serves on the Board of Directors for the Environmental Educators of North Carolina. She educates over 5,000 students a year for schoolyard programs, summer camps, and other extracurricular activities in the environmental sciences. She has a B.S. in Natural Resources from NC State University and a M.S. in Environmental Education from Montreat College. She lives with her husband in Dallas, North Carolina on her family's produce farm.

Ken Darnell's

BYWAYS AND OLD WAYS

By Scott Carlberg, Contributing Author

Ken Darnell learned from old-timers about the forest. Holdovers from the Great Depression in the West Virginia hills. "I grew up with people who spent time in the forest, took care of the land. People who hunted deer and squirrel. Families who gathered blackberries and picnicked at the creek swimming holes."



Ken and Bender, an English Setter from years back, and a faithful hiking companion. Over time, Ken's dogs have been Rudy, Buddy, Bumper, Bo Derek, Bender, Stonewall, Fidget, and now Molly, a Yorky Poo.

Veterans of the hills knew the importance of the American chestnut. Their families once depended on this tree, valued for its rot-resistant lumber and nutritious nuts.

Ken's treks across the hills sparked his dedication to chestnuts. "I grew up at 2,200 feet on Chestnut Ridge. Peaks, 3,000 feet. The ridge, 75 miles long."

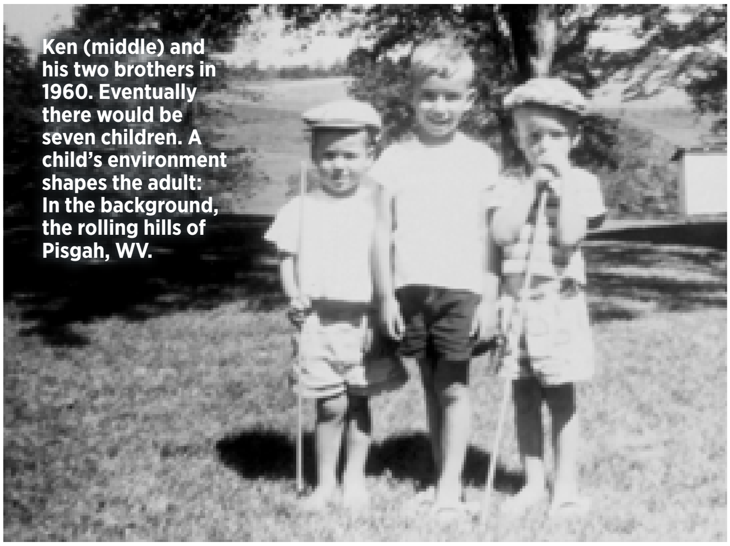
West of the small town of Pisgah, WV, the 12,000+ acre Coopers Rock State Forest was Ken's playground – for this young teenager a classroom. As a boy, he heard stories about the 1930s Civilian Conservation Corps members who built the structures in the park.

"There was lots of room to roam," says Ken, who studied the mountains and the trees. "Huge trees, dead trees, or what some call 'ghost trees' everywhere you looked. Even as a kid I sensed what those trees were about. The conservationist started in me."

Informal study gave way to Ken's formal education in wood science and forestry at nearby West Virginia University. Oddly enough, that education made this outdoorsman something of an indoor professional. His career – cabinet manufacturing for 42 years. He was a plant manager in the kitchen and bath industry for 32 years.

Kentucky is home now for 26 years. He has trekked the countryside throughout Kentucky and West Virginia. He has proof of his treks, too, because he doesn't pitch his old boots. A hiker can get pretty attached to boots.

He brings that kind of dedication to his board duties in the KY-TACF Chapter.



Ken (middle) and his two brothers in 1960. Eventually there would be seven children. A child's environment shapes the adult: In the background, the rolling hills of Pisgah, WV.

Ken, current day, stands in front of an American chestnut hybrid at Eastern Kentucky University's Regional Seed Orchard.

Even at work his early conservation training took hold.

"In the manufacturing plant conservation was part of doing business. Reduce, re-use, and recycle materials," Ken says.

His plant was certified for stringent environmental regulations. "We reduced waste; from making wood cuts the right way, efficiently using the kiln for drying wood, preventing overspray, recycling cardboard, paper, plastics, glass, and even coffee grounds. Sustainability was a state of mind."

Ken has found about 850 American chestnuts on his hikes. "Might say that I have combined hobbies since I retired. I like trees, like to hunt, like to hike, and now I hike and hunt for trees."

A good tree hunter, too, especially in the last 16 months. Ken added 347 observations to TreeSnap, an app to register healthy trees that can be used for research. From the TreeSnap app: "TACF scientists will use the data entered to locate trees for research projects like studying genetic diversity of wild American chestnuts and building better tree breeding programs."

This fits right in for TACF, a partner with TreeSnap developers from the University of Kentucky and the University of Tennessee at Chattanooga.

It's not just trees, though. Remember that Ken studied wood science. Just look around his house. "Chestnut wood is so cool! It is wonderful to handle; lightweight, medium density, and versatile."

Ken uses his wood identification skills to search antique shops for wormy and non-wormy old pieces. "Both are beautiful in different ways, thanks to their ring porous structure, and the chestnut color

Staying Strong, Staying Focused

THE AMERICAN CHESTNUT FOUNDATION®

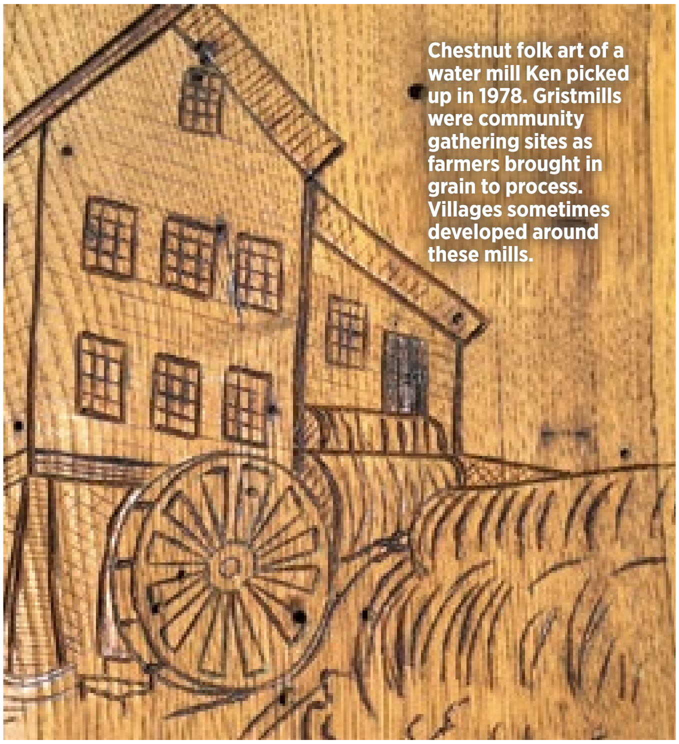
2020 END OF YEAR APPEAL

The American chestnut tree brings to mind images of strength, resilience, and purpose; hopeful words that keep us focused on its restoration. We often embrace those very same virtues ourselves during difficult times.

2020 will most certainly be a year remembered like no other. Despite unprecedented challenges, TACF's community is strong, optimistic, and productive while looking forward to a brighter future.

Thanks in large part to your support, The American Chestnut Foundation has been able to continue focusing on mission success without losing ground. It is your dedication, passion, and faith that carry us through this decades long undertaking and, together, we will succeed in rescuing this iconic and beloved tree!

Continuing our hopeful mission in challenging times.



Chestnut folk art of a water mill Ken picked up in 1978. Gristmills were community gathering sites as farmers brought in grain to process. Villages sometimes developed around these mills.

that comes from the high content of tannic acid. Wormy chestnut has extra character unique for each piece," Ken says.

A chestnut tool made to help farmers or outdoorsmen pick tough knots from rope. From Ken's chestnut collection.

His favorite household pieces show the utility of the wood. One is the simplest. None of Ken's chestnut friends have confirmed its use. It's a 1" diameter by 12" long hand tool, with an awesome dark tannin color, likely carved by a farmer for his specific need – separating knots in ropes, perhaps?

In the KY-TACF Chapter, Ken has been the Festival Committee chair for the past couple of years. "Our team partners with various fall festivals, showing TACF to many folks," he says.

The team also taps social media (Facebook, Twitter, Instagram and group emails) to recruit followers to spread chapter news and develop a pool of volunteers.

Ken's work with TACF builds loyalty to the American chestnut, helps move us ahead in our goal to save the tree. The old timers would be proud!

Autumn

A GREAT TIME OF YEAR TO SEARCH FOR AMERICAN CHESTNUT

By Tom Saielli, Mid-Atlantic and Southern Regional Science Coordinator

Looking for a fun and safe way to spend your free time this fall? Grab your mobile device and download the TreeSnap app. Head for the hills to hunt for wild American chestnuts to support The American Chestnut Foundation's (TACF) breeding program (with proper social distancing, of course). Autumn is a great time to search for these trees – the oppressive summer heat is beginning to wane, fall foliage appears on the horizon, and burs begin to open and drop their seed for harvesting (if you get there before the squirrels)!

John Scrivani, VA-TACF Chapter, displays the fruits of his labor; a nut filled bur he found while hiking in western Virginia. John made sure to bring a pole pruner, a bag for the burs he collected, and TreeSnap to record information, including location and GPS coordinates. Photo by Tom Saielli.

Why should you help locate American chestnut?

Our 3BUR breeding program (Breeding, Biocontrol and Biotechnology United for Restoration) requires a large populous of American chestnut to ensure a robust, genetically diverse population of potentially blight-tolerant trees. We also need to conserve American chestnut germplasm in conservation orchards. Finally, 'chestnut hikes' are a fun and healthy activity for citizen scientists of all ages and abilities.

Why is autumn a great time to search for American chestnut?

After receiving the appropriate permission from landowners, Autumn is the time to collect viable chestnut seeds. Depending on your location, seeds could be collected from late September to early October. Timing is important because when the burs fall and open, the nuts are a delectable treat for wildlife. Finally, be sure to flag trees for later collection of twigs, or scion, for further research use.

TIPS FOR FINDING WILD AMERICAN CHESTNUT

❶ Head for the hills

American chestnut is an upland species, so searching hilly country will improve your odds. The most abundant sites will be in the Appalachian Mountains (the core range of American chestnut), but you can find them at low elevations too, as long as the terrain is hilly. Heading upslope is the trick. Avoid searching river bottoms and cool coves.

❷ Familiarize yourself with chestnut habitat

OAK-HICKORY FOREST

- Soils are moist, but well-drained (not mesic)
- Typically hilly or mountainous
- Upland species

Community type:

- Chestnut oak (the most common co-occurring species)
- Other oaks, including red oaks & white oaks
- Hickory
- White pine
- Mountain laurel
- Sassafras
- Sourwood
- Blueberry

American chestnut predominantly inhabits oak-hickory forests, typically with an abundance of chestnut oak. Look for other common species associated with chestnut, including sassafras, white pine, sourwood, mountain laurel, and blueberry. You are less likely to find chestnut in mesic sites, dominated by maples, birch, ash and carpets of ferns. Red maple, poplars, and beech are overlapping species and can be found growing in a variety of habitats, alongside chestnut as well, but also in cool wet sites, so they are not good indicators of chestnut habitat.

❸ Start with what you know

Look at the American chestnut range map. Where in the chestnut range are you likely to hike or take country drives? Finding hilly, forested areas with trails or backcountry roads is easiest when you are familiar with the region, so start with what you know.

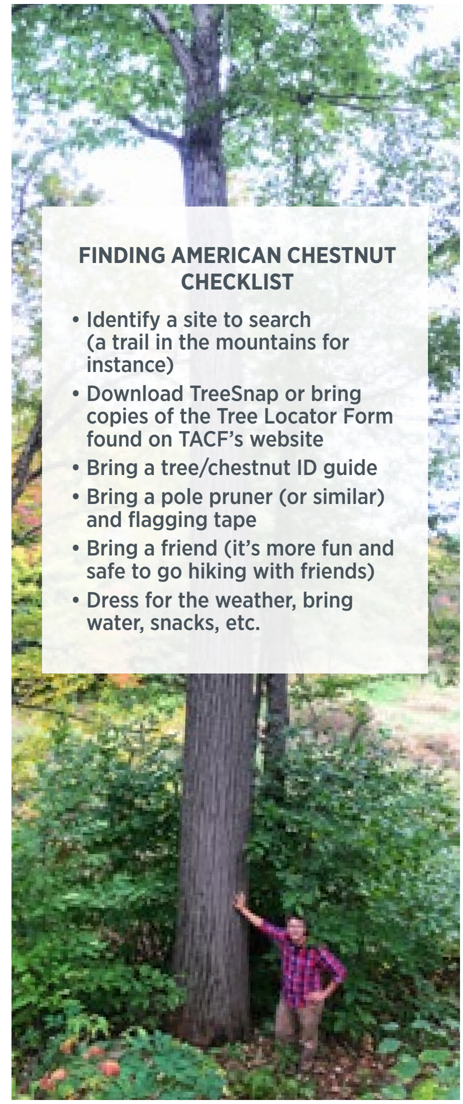
The natural range of the American chestnut (*Castanea dentata*) extended over 200 million acres from Maine to Mississippi.

❹ Additional pro tip

If you know of any sites that have been released in the last decade or so, you may find flowering trees and be able to harvest seeds. "Released" means there has been an opening in the canopy. This could result from a fire, windstorm or cutover, or perhaps along roads or power lines.

❺ Be prepared

Remember to download the TreeSnap app and bring all necessary supplies.



FINDING AMERICAN CHESTNUT CHECKLIST

- Identify a site to search (a trail in the mountains for instance)
- Download TreeSnap or bring copies of the Tree Locator Form found on TACF's website
- Bring a tree/chestnut ID guide
- Bring a pole pruner (or similar) and flagging tape
- Bring a friend (it's more fun and safe to go hiking with friends)
- Dress for the weather, bring water, snacks, etc.

❻ Be safe

Chestnut hikes are COVID-safe, healthy, and fun. However, during the pandemic, it is important to avoid carpooling with non-family members. Go with friends but maintain social distancing and/or wear masks. Remember, your safety and the safety of others is most important.

What to do if you find wild American chestnuts

Document your find in TreeSnap and check for viable seed, which have a plump and smooth appearance. Record location and other relevant information, such as the presence of stump spouts. Store the seeds in a cool, dry place. Contact your state chapter representative or TACF regional science coordinator about your find.

TreeSnap

DOWNLOAD THE TREESNAP APP

- Whenever you find a wild chestnut, use your app to record it
- Open the app and click on "American Chestnut"
- Take lots of pictures – especially of the leaves
- Record data and upload your entry
- It will work even in areas with no service

If the trees are not producing seed, we still want to know about your discovery. Flag the tree(s) and record the location. We may later ask you to go back and collect dormant twigs for grafting, or we may try collecting rooted stump sprouts so we can preserve their DNA.

Questions? Contact your local TACF state chapter or regional science coordinator. Contact information can be found on our website at acf.org.

Happy Hunting!

THE AMERICAN CHESTNUT FOUNDATION®

Give the Gift of Membership

When you give a gift membership to support The American Chestnut Foundation (TACF), you share a story of history and hope. You bolster the promise of future forests thriving with American chestnut once more.

With your one-time gift, your recipient can enjoy a year of engaging news from TACF's award-winning *Chestnut* magazine, member access to the annual wild American seedling sale, and affiliation with their local chapter to get involved at the grass roots level. You may add a personalized message that will be sent along with your heartfelt gift – a gift that not only helps restore the iconic American chestnut tree to its native range, but will benefit our ecosystem and future generations.

Your generosity provides invaluable support to TACF. Thank you!

MEADOWVIEW

MEADOWVIEW RESEARCH FARMS

Using Transgenic Pollen

By Eric Jenkins, Meadowview Tree Breeding Coordinator

Many of you have read or heard about the transgenic American chestnut developed by researchers at the SUNY's College of Environmental Science and Forestry. Researchers inserted a gene called OxO into American chestnut. This gene encodes the oxylate oxidase enzyme that detoxifies oxalic acid produced by the chestnut blight fungus, thus greatly enhancing the blight tolerance of the tree.

While American chestnuts containing the OxO gene are still highly regulated, TACF has a USDA permit to use some of that pollen to perform pollinations on trees at Meadowview Research Farms. This year, farm staff pollinated American backcross hybrids that have resistance to *Phytophthora cinnamomi*, a pathogen that causes root rot. Some of the seed that come from these crosses will have resistance to root rot and chestnut blight, the two major diseases that decimated American chestnut. We also pollinated wild-type American chestnut trees in our Germplasm Conservation Orchard.

PHOTO 1: A view of the burs almost four weeks after pollination.

As with anything subject to regulation, there are procedures that must be followed. The extensive protocols are to ensure any materials containing the OxO gene are under the control of farm staff at all times.

Prior to pollination, we enclose the flowers in fabric bags. These bags are much larger and sturdier (**Photo 1**) than the traditional paper pollination bags. They also have a window so that flower development can be observed. After pollination, we enclose the bags within wire cages to protect the developing burs from wildlife (**Photo 2**). The bags and wire mesh weigh more than standard pollination bags, so a sturdy branch must be selected.

PHOTO 2: Eric Jenkins (article author) encloses the pollination bags in mesh cages.

PHOTO 3: Pollination bags sealed in protective wire mesh cages.

In addition, any other flowers on the tree not enclosed in bags and cages must be removed. We return to the tree weekly to see that the cages and bags are intact (**Photo 3**). If any are damaged or the branch has broken, the bags are immediately removed and all material inside is devitalized (either incinerated or autoclaved). We also monitor an area 8 meters around the drip line of the tree for any volunteer seedlings and will continue this practice for two years after pollination. Any seedlings found must be removed and devitalized.

During harvest, the part of the branch enclosed within the wire cage is cut from the tree and returned to the lab where the seeds are extracted. Seeds are stored in a locked facility separate from our traditionally bred hybrid seeds. All material from the harvest, except the seed, is devitalized. In adherence with regulations from the USDA, we take these precautions to prevent inadvertent escape of transgenic seeds and other plant material.

Approximately half of the seeds harvested will contain the OxO gene and a simple lab test is used to determine which seeds inherited the gene. A small piece of the seed is extracted with a hollow needle and placed in a vial containing the indicator solution. A change in color from clear to blue indicates the gene is present. If the solution remains clear the gene is absent (**Photo 4**). The non-OxO seeds can be used to grow seedlings employed as controls when the resultant seedlings are inoculated with blight.

PHOTO 4: The change in color of the solution from clear to blue indicates the presence of the OxO gene.

PART 3
OF A 3-PART SERIES

Safety Tests
ON TRANSGENIC AMERICAN CHESTNUT

By Andy Newhouse, SUNY's College of Environmental Science and Forestry

Previous articles in this series have described safety of transgenic American chestnuts to people and wildlife; this final installment will cover safety to other plants and fungi. Studying these interactions is uniquely important for transgenic trees. If the transgenic American chestnut is approved for distribution, it will be the first time a transgenic plant is intentionally grown in the wild, so interactions with wild plants and fungi are being carefully examined.

Since genetically engineered crop plants are common and highly regulated, safety tests on transgenic food products and animal interactions are common and relatively well-understood. However, evaluations involving environmental interactions in a forest ecosystem are relatively new, so we need to be both careful and creative in how we perform these tests. And since the purpose of the transgene is to provide tolerance to a fungal pathogen, it is especially important to understand how it could affect beneficial fungi.

The first step in looking at transgene interactions with fungi involves understanding how the transgene works. The oxalate oxidase (OxO) enzyme in 'Darling 58', which is found in wheat and a variety of other plants, does not kill or repel fungi – instead, it breaks down a toxin (oxalic acid) that is produced by the blight fungus. This suggests that OxO should not be directly harmful, even to the blight fungus itself, or to other fungi or life forms. In fact, oxalic acid is toxic to both people and other plants, so OxO can actually be considered an anti-toxin. (See Part 1 of this series in the 2020 winter issue, volume 34.)

Going beyond the logic arguments about the mechanism of OxO, we have conducted several types of experiments to look at chestnut roots and mycorrhizal fungi. Mycorrhizae are relationships between soil fungi and plant roots (**Figure 1**). These are typically "mutualistic" relationships, since they are beneficial to both organisms. If a new type of tree were unable to form mycorrhizae, we probably would not want to use it for wild restoration. But repeated tests[1, 2, 3] on OxO-expressing American chestnuts have shown that transgenic tree roots form mycorrhizae which are just as prevalent and diverse as those found on wild-type trees. These tests have taken place in both greenhouse and natural environments with similar results: in the most recent test, for example, more than 95% of the root tips we observed formed healthy mycorrhizae on both Darling 58 and non-transgenic controls.

In order to study a different environmental interaction that occurs in the soil[2], we started with seeds from other wild plants that are commonly found in American chestnut habitats. These included grasses, wildflowers,

Figure 1

Chestnut root tips encased in two types of mycorrhizal fungi. These fungi can help the tree access water and nutrients, in exchange for energy in the form of sugar from photosynthesis.

shrubs, and trees. We germinated these seeds in potting soil containing crushed chestnut leaves of various types: transgenic American chestnut; a related non-transgenic control; backcross; Chinese; F_1 hybrid; an unrelated American chestnut; and a no-leaf control. We counted seedling germination in each type of chestnut leaves (**Figure 2**), and recorded the total mass of all seedlings grown in each leaf type. There were only two statistically significant differences among leaf types in the whole study: first was dry mass of one wildflower type, which was slightly lower in B_3F_3 leaves than Darling 58 leaves. (Note – while this difference was statistically significant, we shouldn't assume that B_3F_3 leaves inhibit plant growth. Normal growth of this flower in F_1 hybrid and Chinese leaves would suggest we're just seeing a range of flower growth, rather than a real effect due to the B_3F_3's genetic background.) The other significant difference was in the number of pine seedlings that germinated in the unrelated wild-type American chestnut leaves compared to the no-leaf control treatment, but pine germination was not unusual in either Darling 58 or B_3F_3 leaves. An unrelated experiment in three of our field plots also looked at plant growth near various types of chestnuts: again, there were no differences in plant growth based on the type of chestnut that was growing nearby.

The final interaction to be described in this article also involves chestnut leaves after they fall from the tree. If you can picture a deciduous forest in autumn, you probably understand the ecological importance of leaf decomposition! One question about transgene safety involves how long the transgene product (the OxO enzyme, in this case) remains biologically active. Of course, OxO must be active while it's in the tree; that is what breaks down the toxin and imparts blight tolerance. But what happens in leaves or branches once they fall from a transgenic tree? We used a simple enzyme activity assay (see article by Thomas Klak on page 30) to test enzyme activity in the fall while leaves were still attached to trees, and then at a series of time-points after they had been removed. We determined that it is possible to preserve OxO activity in leaves for several months, but only if the leaves are carefully packed in plastic bags and immediately stored in a freezer. In natural conditions, where leaves are exposed to temperature fluctuations and dry relatively quickly, OxO activity ceased as soon as the leaf dried or turned brown (**Figure 3**). This occurred after about a week during normal outdoor conditions in our tests. This suggests that the presence of OxO in leaves should not substantially affect decomposition and associated nutrient cycling processes.

More detail on these tests and many others are described in our petition to the USDA for nonregulated status of the Darling 58 American chestnut, which is now publicly available. To learn more about how you can support this important project, visit The American Chestnut Foundation's public comment period webpage at acf.org.

Figure 2

Roots of a wildflower growing through a chestnut leaf.

Figure 3

Chestnut leaves at various stages of decomposition. The OxO enzyme is no longer active once the leaf dries or turns brown (middle leaf).

FOOTNOTES

[1] https://doi.org/10.1128/AEM.02169-14.
[2] https://doi.org/10.3389/fpls.2018.01046.
[3] https://pqdtopen.proquest.com/doc/1418032936.html?FMT=ABS.

Developing Marker Assisted Selection

FOR BREEDING BLIGHT-RESISTANT HYBRID CHESTNUT

By Nicholas R. LaBonte, University of Illinois[1]; Aziz Ebrahimi, Purdue University[2]; and James R. McKenna, U.S. Forest Service[3]

Marker assisted selection (MAS) is a tool that plant breeders use to identify desirable progeny from an early age by associating DNA markers with desirable phenotypes (Collard & Mackill 2008). For tree breeding, MAS can vastly improve efficiency and save years or decades to achieve breeding goals. In a TACF-funded study begun in 2014, we prepared whole-genome assemblies of 24 Chinese chestnuts (*Castanea mollissima* [*Cm*]), interspecific hybrids, and American chestnuts (*C. dentata* [*Cd*]) by assembling millions of short DNA fragments to a Chinese chestnut draft reference genome (Fang et al. 2013, Staton et al. 2015). We were able to identify millions of "single-nucleotide-polymorphism" (SNP) markers in these genomes. We analyzed patterns of DNA sequence variation in Chinese and American chestnuts in gene regions associated with blight resistance based on past chestnut genomic research (Kubisiak et al. 1997, 2013).

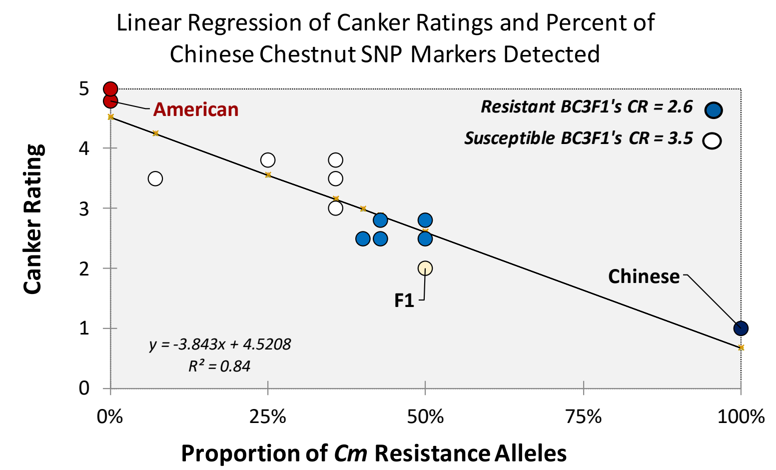
Bruce Wakeland, TACF board member, pollinating Indiana American chestnuts in June 2017.

To further narrow these SNPs, we identified regions throughout the genome where resistant and susceptible Chinese trees had different alleles at the same SNP marker. For such differing SNP markers, we were able to examine the suggested function of their associated genes based on Barakat et al. 2009, 2012, to select 15 novel SNPs for MAS for hybrid chestnut blight resistance breeding.

Validation of these SNP markers began with another TACF grant a few years later in 2017 where we tested a variety of hybrid chestnut trees from the IN-TACF Chapter and a sample of resistant and susceptible BC_3s from breeding orchards at TACF's Meadowview Research Farms in Virginia. Phenotypic resistance scores were based on the canker rating of 4- to 16-year-old trees in the field, previously inoculated with blight isolates *SG* and *Ep 155*, and in some cases based on variation with natural blight infection.

In our first test, we extracted DNA from dormant twigs of American, Chinese, F_1, and resistant and susceptible BC_3F chestnuts from the IN-TACF Chapter

Figure 1



Correlation of blight-resistant SNP Markers with 6 to 16 year IN-TACF BC_3F_1 progeny grown in Starke, Tippecanoe, and Washington Counties of Indiana. The F_1 hybrid is a novel natural cross of American × Chinese chestnut from Grant Co., Indiana. The Chinese is 'Hort 14' that was purchased about 30 years ago from Empire chestnut. American chestnuts were both grafted clones from the Duke Energy Orchard at the Martell Forest, West Lafayette, IN.

Table 1

Sample ID	Spp. Genotype	Orchard	Block	R-T	CR*	SNP Locus: Lgc_338 4:b37	LGF_g18 03:b89	LGF_g18 04:b83	LGF_g2785 :b104	LGG_g365 7b:b35	LGL_g895 3a:b109	LGL_g8953 b:b125	Cm dosage	Loci	Cm/ locus
Sigma Chi	CC	Martell	na	na	R+	Cm	Cm	Cm	Cm	Cm	Cm	Cm	12	6	2.00
East barn	CC	SIPAC	na	na	R+	Cm	Cm	Cm	Cm	Cm	Cm	Cm	14	7	2.00
West barn	CC	SIPAC	na	na	R+	Cm	Cm	Cm	Cm	Cm	Cm	Cm	14	7	2.00
17-2.1	F1	Martell	na	na	R	–	Cm/Cd	Cm/Cd	–	Cm/Cd	Cm/Cd	Cm/Cd	5	5	1.00
17-2.2	F1	Martell	na	na	R	–	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	6	6	1.00
17-2.3	F1	Martell	na	na	R	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	7	7	1.00
17-4.1	AC	Duke	na	mix	S+	Cd	Cd	Cd	Cd	Cd	Cd	Cd	0	7	0.00
17-4.2	AC	Duke	na	mix	S+	Cd	Cd	Cd	Cd	Cd	Cd	Cd	0	7	0.00
17-4.3	AC	Duke	na	mix	S+	Cd	Cd	Cd	Cd	Cd	Cd	Cd	0	7	0.00
Line 4A	B1F1	SIPAC	1	R1-T17	"R"	–	Cd	Cd	Cm/Cd	Cd	Cd	Cd	1	6	0.17
Line 4A	B1F1	SIPAC	1	R1-T18	"R"	Cd	Cd	Cd	Cm/Cd	Cd	Cd	Cd	1	7	0.14
Line 4A	B1F1	SIPAC	1	R2-T20	"R"	–	Cm/Cd	Cm/Cd	Cd	–	Cm/Cd	Cm/Cd	3	5	0.60
Line 4A	B1F1	SIPAC	1	R2-T15	"S"	Cm/Cd	Cd	Cd	Cm/Cd	Cm/Cd	Cm	Cm	3	7	0.43
Line 4A	B1F1	SIPAC	1	R2-T10	"S"	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	Cd	Cd	Cd	4	7	0.57
Line 4A	B1F1	SIPAC	1	R2-T9	"S"	Cd	Cd	Cd	Cd	Cm	Cm/Cd	Cm/Cd	4	7	0.57
Line 1A	B3F2	SIPAC	1	R3-T10	R	Cm/Cd	Cd	Cd	Cm/Cd	Cm/Cd	Cm/Cd	Cm/Cd	5	7	0.71
Line 1A	B3F2	SIPAC	2	R6-T12	R	Cm/Cd	Cd	Cd	Cm/Cd	Cd	Cm/Cd	Cm/Cd	4	7	0.57
Line 1A	B3F2	SIPAC	1	R1-T20	R	Cm/Cd	–	Cm/Cd	Cm/Cd	Cd	Cm/Cd	Cm/Cd	5	6	0.83
RL2 x GL367	B3F1	Lugar	na	R21-T23	R	Cm/Cd	Cd	Cm/Cd	Cd	Cd	Cd	Cd	2	7	0.29
DOE x CH526	B3F1	Lugar	na	R1-T53	R	Cd	Cd	Cm/Cd	Cd	Cm/Cd	Cd	Cd	2	7	0.29
RL3 x GR97	B3F1	Lugar	na	R17-T4	R										
TACF '12-F3	B3F3	Parke Co.	ns	na	R?	Cm/Cd	Cm	Cd	Cd	Cd	Cd	Cd	3	7	0.43
TACF '12-F3	B3F3	Parke Co.	ns	na	R?	Cd	Cd	Cm/Cd	Cd	Cm/Cd	Cm/Cd	Cm/Cd	4	7	0.57
TACF '12-F3	B3F3	Parke Co.	ns	na	R?	Cd	Cd	Cd	Cd	Cd	Cd	Cd	0	7	0.00

*CR= Field canker rating: R+ Highly resistant; R Resistant; "R" 2-month SG canker was small; R?; "S"; S+

Additional genotypes and trees from Indiana tested with 7 SNP blight resistance markers illustrating the two alleles at each locus. *Cm* = Chinese chestnut; *Cd* = American chestnut. *Cm* or *Cd* alone indicate a homozygous state where both alleles are the same; *Cm/Cd* = the heterozygous state where one Chinese and one American allele occur at that locus.

Table 2

Pools	Genotype	No. Trees Pooled	State Growing Trees	Field Resistance to Blight	American (Cd) allele fraction of 15 SNP Markers
American	AC	7**	IN	Highly Susceptible	1.00*
F1	AxC	7	IN	Very Resistant	0.50
Chinese	CC	10	IN	Highly Resistant	0.00
TACF-R-B3F3	B3F3	8	VA	Resistant	0.69
TACF-S-B3F3	B3F3	10	VA	Susceptible	0.93
IN-TACF -R- JWSF - TACF- B3F3	B3F3	10	IN	Resistant	0.90
IN-TACF -S- JWSF - TACF- B3F3	B3F3	10	IN	Susceptible	0.93
IN-TACF -R-JWSF '3A' - B3F1	B3F1	1	IN	Resistant	0.78
IN-TACF -JWSF '3A' & other R B1- B3's	B1F1,B3F1,B3F2,B3F3	10*	IN	Mixed Resistance	0.84**

* Estimated from previous data. Americans have shown no Cm resistance alleles; conversely, only susceptible Cd alleles at these 15 loci.

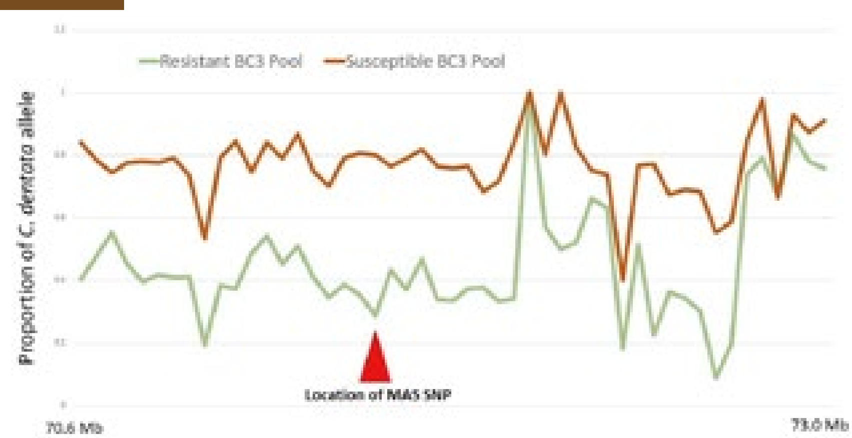
** Includes the JWSF '3A' selection which alone scored 0.78 (listed above).

Pooled samples of known chestnut species and the resistant and susceptible trees from Indiana (IN) and Virginia (VA) along with an individual highly resistant Indiana tree (JWSF 3A) and a mixed pool with this tree and a wide variety of others.

breeding program. The results were very promising and showed the expected results for the pure American, Chinese, and F_1, and the resistant BC_3s had more Chinese alleles than the susceptible BC_3s. Here we found a strong correlation with both increasing resistance and increasing SNP markers (**Figure 1**).

At each SNP marker locus, two alleles exist which can be *Cd/Cd* = American; *Cm/Cm* = Chinese; and *Cm/Cd* for an F1 hybrid. Thus, when multiple markers are run, one can add up the proportion of total *Cm* alleles at all loci. For our second test, we sampled more Chinese, more F_1s, and included B_1F_1s, B_3F_2s and B_3F_3s (**Table 1**). Ultimately, the higher the value of "*Cm*/locus" should reflect the higher resistance of the tree. Through a "small stem assay" (Powell et al., 2007), conducted in the field in 2017, we selected three "resistant" and three "susceptible" B_1F_1s. Unfortunately, these didn't test out as expected and our resistant selections had fewer *Cm* alleles than our susceptible selections.

Figure 2



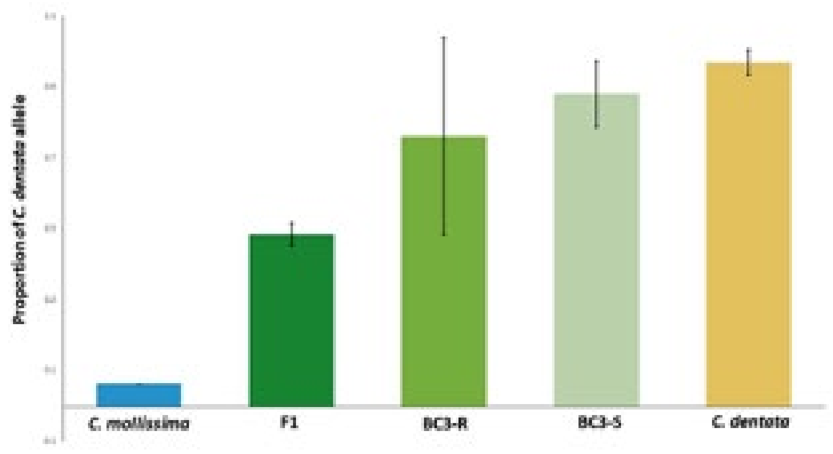
Allele frequencies in resistant and susceptible BC_3F_2 pooled whole-genome sequences on a section of linkage group L. A region with a putative blight resistance gene shows a lower frequency of *C. dentata* alleles in this region and conversely, a higher proportion of *C. mollissima* resistance alleles.

Next, we compared both resistant and susceptible BC_3F_3 families and individuals from an Indiana B_3F_3 progeny test planted in 2014 as well as selections that had been evaluated at Meadowview, Virginia and shipped to us. The Indiana trees were planted next to a 2003 B_3F_1 breeding block where chestnut blight had become endemic and the disease began naturally infecting these trees by their second year. We rated all 2014 trees in the 2017-18 winter for blight to calculate family blight incidence and severity ratings to determine resistance. The Meadowview trees had been inoculated several to four years prior and canker sizes were measured and breeding values determined. From both groups, we tested 8 to 10 resistant and 10 susceptible trees with 11 SNPs (**Table 2**).

We ran 11 SNPs for each individual tree but had problems with the output for several trees and several markers. We decided to redo this test but "pool" each group instead of running each individual tree again along with several other pools and individual trees. Pooled sequencing allows for a bulked-segregant-like analysis of breeding materials so that more individuals can be tested with fewer sequencing costs and time; individuals from pools with high resistance can then be tested individually to find the most resistant genotypes while pools with little resistance can be rogued out.

The SNPs showed just 3% difference between resistant and susceptible Indiana trees while in contrast, the Meadowview selections showed a 24% difference between resistant and susceptible trees. For the Indiana trees, relying on natural infection and just four years in the field, we assessed resistance too soon and made poor selections. In contrast, from the 2003 neighboring block, our most resistant B_3F_1 in Indiana (JWSF 3A), scored 15% more resistance based on these SNP markers. This tree has maintained its original inoculated trunk from 2008 until the present day and is still exhibiting good resistance. In contrast, the Meadowview resistant selections had markedly more *Cm* alleles and demonstrate the preferred approach of controlled inoculations.

Figure 3



Average proportion of American chestnut (*C. dentata*) alleles observed over 12 putative blight SNP markers for n=2 *C. mollissima*, n=4 F1 hybrids, n=4 *C. dentata*, n=41 resistant BC_3 chestnuts, and n=33 susceptible chestnuts. Error bars are standard deviations.

Finally, we pooled every resistant and susceptible BC_3 and sequenced the whole genome of both groups to see how all 15 SNPs differed among the two groups. A SNP from linkage group L, within a predicted gene similar to known plant disease resistance genes, showed an allele frequency dramatically skewed towards the *C. mollissima* allele in resistant backcross trees (**Figure 2**). This gene also showed a difference in expression in a study of RNA sequences in developing blight cankers (Barakat et al. 2012). As expected, one of these regions corresponded to the potential blight resistance gene on LGL. In all tests, *Cm* alleles have proven to be strongly associated with blight resistance and the corresponding *Cd* alleles have been strongly associated with susceptibility (**Figure 3**).

These SNP markers should help breeders select more durable blight resistance. Our study demonstrates a way to use high-throughput, low-cost (~$2/sample/marker), whole-genome sequencing for identifying low-cost markers for marker assisted selection, especially well-suited for backcross interspecific hybrid breeding.

FOOTNOTES

[1] University of Illinois, Champaign Urbana, IL.

[2] Department of Forestry and Natural Resources, Purdue University, West Lafayette, IN.

[3] USDA Forest Service – Northern Research Station, West Lafayette, IN

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Comparison of Four Methods

FOR NON-DESTRUCTIVE TESTING OF CHESTNUT SEEDS FOR OXO GENE ACTIVITY*

By Thomas Klak, Woon Yuen Koh, Tyler Riendeau, and Andrew Grammas, University of New England, Biddeford, Maine

Figure 1: Chestnuts sliced, sealed with silicone, and waiting to dry before being returned to their uniquely ID'd tea bags.

*We would like to thank Andy Newhouse, researcher at SUNY College of Environmental Science and Forestry, and Brian Roth of the ME-TACF Chapter, for their helpful review.

Chestnut restoration efforts that include genetic engineering will require a test to detect the activity of the OxO gene, since about fifty percent of offspring of crosses between transgenic pollen and wild-type mother trees inherit the transgene.

In previous years, practitioners at SUNY's College of Environmental Science and Forestry (ESF) have sampled either a seedling leaf or a core from the seed for OxO activity. Compared to testing the seeds, the seedling test takes more time and resources (because nuts have to germinate), and may be harder to interpret due to leaf pigmentation or other differences. By either method, the sample is exposed to an oxalic acid test solution in a small test tube, and if the OxO gene is active, hydrogen peroxide is emitted, coloring the solution black. This screening process allows for just seeds and seedlings with the OxO gene to be effectively deployed in restoration programs.

Sampling seeds directly would be beneficial as it could allow us to focus efforts on seeds we know to be transgenic, rather than germinating everything before screening. We therefore tested four non-destructive methods of sampling tissue from a seed's cotyledons. The first two methods involve extracting a core sample with a 2mm bone marrow needle (**Figure 2**). The difference is that one method involves painting the hole with aquarium-grade silicone to keep pathogens out, which is the method ESF has used of late. The other method leaves the hole uncovered. In the other two methods, a small knife is used to extract a slice from the cotyledon. Again, the two slicing methods differ on whether or not the wound is sealed with silicone (**Figure 1**). Which of the four methods is most efficient to deploy, while minimizing damage to the seed that could affect germination and growth?

Figure 2

A chestnut cotyledon core extracted with a 2mm bone marrow needle.

Methods and Results

At this point, potentially-transgenic seeds are rare and especially valuable. We therefore used non-transgenic seeds from 19 wild-type mother trees in this sampling-methods test. We cold-moist stratified all seeds for approximately 2.5 months after harvest before testing. We weighed and sampled the seeds over a two-day period in late January (**Figure 3**), and then indoor-sowed all seeds in early February, 10-12 days after sampling. Thirty-eight days after sowing the chestnuts, we recorded which seeds had germinated and measured the seedling heights (**Figure 4**).

As shown in the **Table 1**, all five treatments obtained germination rates around 90%, which is typical for chestnuts. Chi-square test and Fisher's Exact test reveal that the germination rate for each treatment is not statistically different than that of the control group.

We noticed that mother trees contributed to the variability of the whole nut weight, percent of the nut removed by the four sampling methods, and 38-day height. We therefore grouped seeds from the same mother tree into "blocks" in order to ensure any differences were due to the treatment instead of the mother tree. We then performed the ANOVA test with blocking and multiple comparisons for testing the equality of means for the above variables.

Statistical results reveal that the weights of chestnuts in the five treatments were not statistically different from one another (all were approximately 4 grams; Table 1). The mean seedling height for Slice No Silicone was statistically different from both Slice Silicone and Core Silicone, but none of the four sampling methods produced seedling heights that were different from the control group. The mean percentage of the nut removed was not significantly different for the pairs Core No Silicone - Core Silicone and Slice No Silicone - Slice Silicone, but was significantly different for the rest of the four pairs of treatments. Slicing removes more cotyledon (~3% of nut mass) than does

Table 1

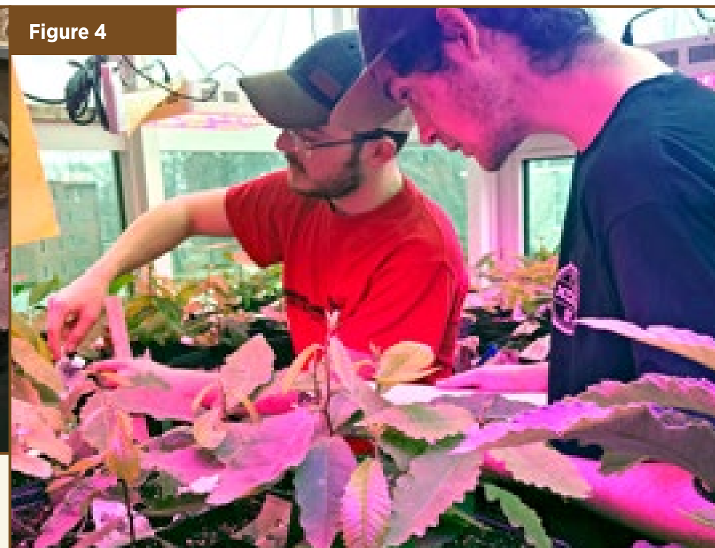
Treatment	Chestnuts Tested & Sowed	Whole Nut Weight (g) Mean (SD)	% of the nut removed Mean (SD)	Sampling Time (sec) Mean (SD)	Chestnuts that Germinated	Percentage Germinated	Height (cm) Mean (SD)
Control	87	4.3 (2.2)	n/a	n/a	80	92%	14.6 (6.1)
Core No Silicone	73	3.6 (1.8)	1.05 (0.42)	45.0 (11.2)	65	89%	14.0 (5.4)
Core Silicone	73	4.2 (2.1)	1.01 (0.44)	47.1 (7.7)	68	93%	13.6 (6.3)
Slice No Silicone	73	4.0 (2.2)	2.81 (1.54)	22.3 (4.7)	71	97%	16.1(7.6)
Slice Silicone	74	3.7 (2.0)	2.86 (1.53)	34.5 (10.8)	64	86%	12.3(5.9)
Total/Overall Mean	**380**	**4.0**	**n/a**	**n/a**	**348**	**92%**	**14.2**

Chestnuts Tested, Whole Nut Weights, Percentage of the Nut Removed, Time to Sample, Germination Rates, and Seedling Height for the Four Sampling Methods and the Control.

Figure 3

From right to left: University of New England students Tyler Riendeau, Andrew Grammas, and Stephanie Kuplast core, weigh, and apply silicone to chestnut seeds.

Figure 4



Students Everett Pierce and Ethan Meyer measure seedling height in the University of New England greenhouse.

coring (~1%), but these weights did not vary according to the presence of silicone. Importantly, as revealed in Table 1, removing more cotyledon did not negatively affect the germination rate nor the seedling height.

How much time did it take to sample chestnuts using the four methods? As mother trees did not contribute significantly to the variability of time required to sample per method, we did not group seeds from the same mother tree into "blocks." The ANOVA test with multiple comparisons was performed to test the equality of mean time required to sample. Results reveal that there was no significant difference between Core No Silicone - Core Silicone, but there was significant difference between all other pairs of treatments. If we get to the point where we need to screen thousands of nuts for transgene presence, efficient use of time and resources will be critical. We found that both slicing methods were on average faster (~22-35 seconds) than the two coring methods (~45-47 seconds).

Conclusion

All four chestnut sampling methods yielded satisfactory results in that they did not negatively affect germination rates or seedling heights compared to the control group. Our study supports the view that practitioners can confidently deploy any of these four non-destructive sampling methods to detect the activity of the OxO gene.

However, our preferred method is Slice No Silicone. It is the fastest of the four techniques, and even though it removes a greater portion of the cotyledon than coring, it yielded germination rates and seedling heights as good or better than the others, including the control group. We recommend Slice No Silicone as an efficient and effective method for future non-destructive sampling of chestnuts when testing for OxO gene activity.

The Reawakening

By Edwin Shuttleworth, TACF Member

My interest in the American chestnut began during childhood in upstate New York when my father told me of his adventures with his older sisters. They would fill shoeboxes with chestnuts found in the woods a short walk from their home. I still search for those wonderful trees when visiting Massachusetts and Connecticut, finding essentially only small coppiced saplings from an old stump. I did find one tree which bore nuts but only sterile ones, some of which I nevertheless keep as mementos in a small glass display box.

Edwin's painting was created entirely from his imagination in 2003. "The Reawakening" was inspired by real life experiences.

Purée de Marrons
(Chestnut Purée)

As many of you know, chestnuts are prized in Europe for their culinary versatility and delicious flavor. We are delighted to share a story of chestnut enthusiast Ariane Wellin, who was born in France to a Russian father and French mother. TACF President and CEO Lisa Thomson met Ariane in Florida earlier this year, and she spoke fondly of her love of a traditional chestnut purée recipe, Purée de Marrons, and declared, "I make the purée every year at Thanksgiving, whether I'm entertaining a house full or am by myself!" She said although the recipe is fairly simple, the labor is in the careful peeling of the chestnuts themselves. Ariane also recalled as a child eating Marrons Glacés, delectable confections where chestnuts are consumed as candies or added to an infinite variety of recipes. Bon appétit!

Yield
6 to 8 servings

Ingredients
8 cups peeled chestnuts

2 stalks celery

1 medium herb bouquet: 4 parsley sprigs, 1/2 bay leaf, and 1/8 tsp thyme tied in cheese cloth

3 cups good brown stock or 2 cups canned beef bouillon and 1 cup of water

3 to 6 Tb softened butter, or butter and whipping cream

Salt and pepper

Pinch of sugar, if needed

Equipment
A 3-quart, heavy-bottomed saucepan

A food mill

Method
Place in the saucepan the chestnuts, celery stalks, and herb bouquet. Pour in enough stock, or bouillon and water to cover the chestnuts by 1 1/2 inches. Simmer very slowly and uncovered for 45 to 60 minutes, or until the chestnuts are cooked through. Do not overcook and allow them to become mushy. Drain immediately; remove celery and herb bouquet. Purée the chestnuts in the food mill, then return them to the saucepan.

Beat in the butter, or butter and cream. If purée is too thick, beat in spoonfuls of the cooking liquid. Season to taste with salt and pepper, and a pinch or two of sugar if you feel it necessary.

*If not to be used immediately, instead of beating in the butter, spread it over the surface of the purée. To reheat, cover and set over boiling water, beating occasionally.

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Sabrina Poulin

Ellie Rudd
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Lisa Pauer

Woodland owners across Appalachia who suffered the loss of the chestnut
From:
Bob Caldwell

Michael Winters
From:
Jo Keim

Ned Yost
From:
Robert E. Reuter

Stan Young
From:
Carol Young

We regret any errors or omissions and hope you will bring them to our attention.

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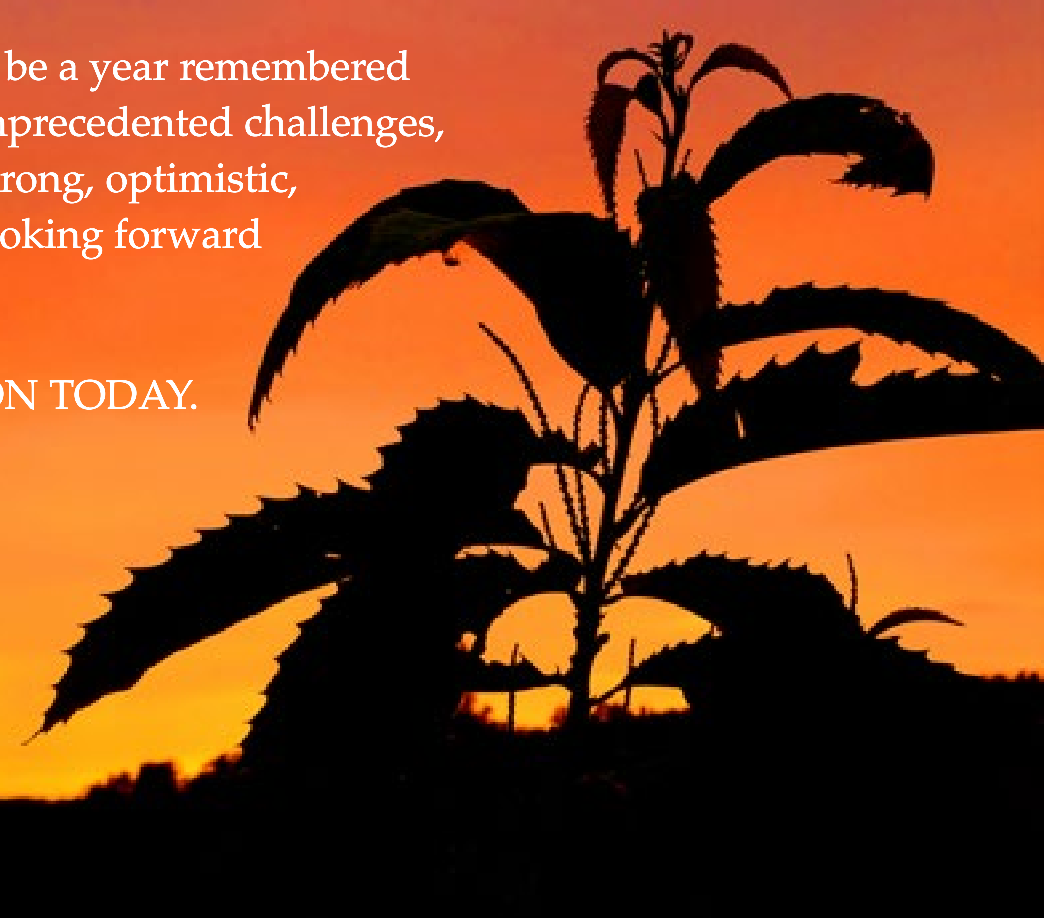

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