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**Special Issue: Chestnut Blight and Blight Resistance;  
Where TACF Stands Today**

# What is Blight Resistance?

By Glen Rea, TACF Chairman of the Board, and Bryan Burhans, TACF President and CEO

This issue of *The Journal* focuses on a core objective of TACF: to develop chestnut trees resistant to the chestnut blight that removed the species as a dominant forest tree over a hundred years ago. But, what is resistance?

Blight resistance can take on a variety of different meanings depending on your point of view. An important distinction is that we cannot develop trees that are *immune* to the blight. No species of chestnut is immune to blight, since all of them can get the disease. However, several species are resistant to the blight; they get the disease, but only mild cases. High levels of blight resistance are found in Asian species of chestnut, and the Chinese chestnut, *C. mollissima*, has the highest levels of resistance that have been measured. TACF is using *C. mollissima* as its primary source of blight resistance.

Since the organization's inception, an important and overarching goal has been to develop American chestnut trees with enough resistance to return the species, *Castanea dentata*, to our forests. The important issue is the *level* of resistance necessary to accomplish this goal.

The native American chestnuts still growing in our forests have functionally ceased to evolve; trees simply can't evolve if they can't reproduce. Evolution through natural selection is nature's own built-in breeding program. Since chestnuts now rarely reproduce in the wild, there is no chance for evolution to work on the existing population.

To bring the chestnut back, we are working to develop chestnut trees with the necessary levels of genetic diversity and disease resistance so that the resulting chestnut *population* can once again evolve in our forests. Restarting the evolution of the American chestnut requires that the trees grow to produce progeny, which in turn produce progeny of their own. Over time, resulting progeny that have the "right stuff" will survive, and those that do not, will die.

The concept of *population* is important. The relative success of individual trees is not a concern, but the resulting success of a local population of trees and the ability of that population to reach sexual maturity and reproduce in the wild is the difference between success and failure. Many individual trees we will plant will not survive. Some will not have enough resistance. Others may die for other reasons; succumbing to chestnut blight is just one cause of death.

We are still in the early stages of testing our Restoration Chestnuts 1.0. After all, we planted our first seedlings just a few years ago. Early results indicate that we have produced chestnuts with varying levels of resistance, but only time will tell if the resistance in our current test trees will persist as they grow older and if this resistance will suffice when the trees are actually growing in the forest instead of an orchard.

We also anticipate that the proportion of highly resistant Restoration Chestnuts 1.0 coming from our Legacy Tree orchards will increase as we cull inferior parents and increase the collective level of blight resistance. Because of the ongoing culling in the Legacy Tree orchards, the Restoration Chestnuts we produce in any one year really only provide a snapshot of the progress in our breeding efforts.

These are exciting times for the American chestnut. Restoring the tree to our forests is similar to climbing a tall mountain. We have scouted out our best route to the summit. Now we must climb.

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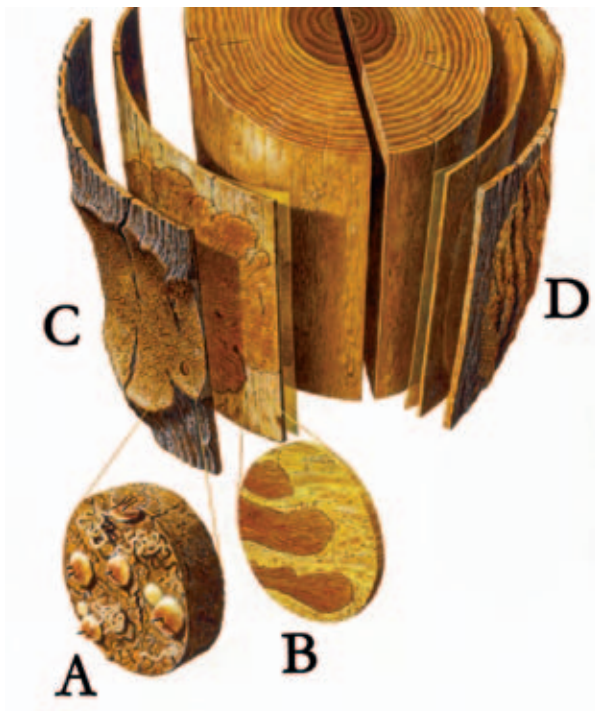
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# Introduction to Chestnut Blight

by Anna Huckabee Smith



Blight canker on an American chestnut tree.



Graphic representation of chestnut blight (*Cryphonectria parasitica*).  
Image courtesy of National Geographic Society

When the blight was first identified in 1904, science knew little about this new disease, and the best efforts of the day to stop its spread were largely ineffective. Today, much more is known about the chestnut blight and the fungus pathogen that causes it, the aptly named *Cryphonectria parasitica*. Yet even in this day of genetic analysis and molecular biology, much more needs to be discovered as the whole story of chestnut blight is only revealing itself a little at a time.

*Cryphonectria parasitica* is a member of the Ascomycetes group of fungi, many of which are parasitic, including Dutch elm disease and oak wilt fungi. The same fungus that attacks American chestnuts also devastated Ozark chinquapin and is mildly pathogenic on Allegheny chinkapin and live post and scarlet oaks. *C. parasitica* can infect any part of the trunk or limbs, gaining access into the tree's living bark tissues through wounds. A common entry point is at a branch node where the constant sway and growth of the limb causes splits in the bark. Once the fungus penetrates the bark, filaments that are threadlike in appearance fan out through the tree (B). A raised (D) or sunken (C) canker is formed. When the infection reaches down to the vascular cambium and functional xylem and phloem, transport of nutrients and water are cut off to areas above and below the canker, growth is restricted, leaves turn brown and eventually, the stem/trunk above the canker dies.

When the fungus prepares to reproduce, it erupts through the older portions of the canker as bright orange or yellow fruiting pimples called stromata (A). Each is the size of a large pin head. Two types of spores are produced in stromata: sexual spores called ascospores, which are forcibly ejected from black, vase-like structures called perithecia, and asexual spores called conidia, which ooze out of round, fruiting bodies called pycnidia after rains.


Conidia can hitch a ride to their next victim on the bodies of birds and insects or be carried in water droplets, while ascospores are windborne.

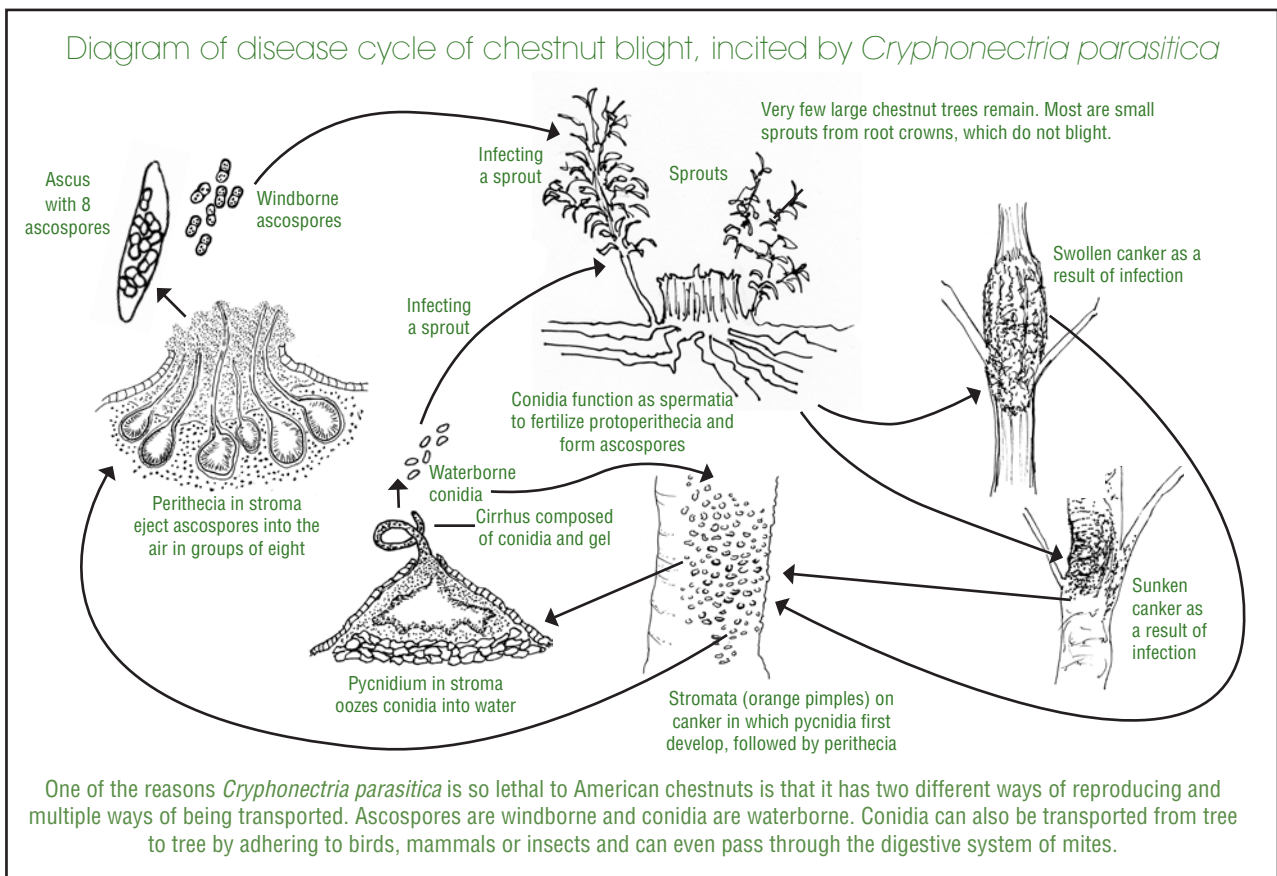
Stromata can be moved closer to their next victim by unsuspecting humans collecting firewood or transplanting trees (the fungus was originally introduced into the United States on chestnut nursery stock imported from Japan). Because the fungus has a mixed mating system, it is able to both self-fertilize and outcross (see graphic of the life cycle of the *C. parasitica* below).

A mature American chestnut that has become infected may have one or many cankers deforming its bark. Most succumb to the disease anywhere from two to ten years later. Saplings rarely last a year. However, because the fungus does not affect the root system, the long-lived American chestnut can produce sprouts from stumps for many years.



Orange stromata on a blight canker  
Photo by John Stempa

Researchers have been looking for a way to not only breed resistance into American chestnut but also, at the same time, to weaken the blight fungus. Infecting the fungus with a virus to reduce its virulence, using agrochemical treatments, and even altering the fungus's genes have all been considered. Hopefully science will one day find a way to halt or at least lessen the impact of this forest pathogen. 





Robert Scarborough plants another row of advanced seeds at Meadowview Research Farms. Orchards at Meadowview contain over 55,000 chestnut trees that are part of the breeding program. Photo by Jeff Donahue

Aldo Leopold, who is often called the father of wildlife ecology, once commented, “That the situation appears hopeless should not prevent us from doing our best.” Three decades ago, facing a situation that some of the era’s finest minds had declared hopeless, The American Chestnut Foundation (TACF) set out on an epic journey to bring the vanishing American chestnut back to the eastern woodlands of the United States. If Leopold were alive today, he likely would applaud the accomplishments of TACF. But what exactly has TACF accomplished? Here on the doorstep of success, two of the most challenging questions for TACF to answer are: Where are they in the breeding process? and, Do they have a blight-resistant tree yet?

To understand where TACF’s breeding program stands today, it helps to revisit the history of the program. Beginning in the early 1920s the USDA and The Connecticut Agricultural Experiment Station began breeding American chestnut trees with Asian chestnut species that were naturally resistant to the fungus that caused blight. These early breeding programs were shut down in the 1960s after they failed to produce blight-resistant trees that also looked, grew, and produced nuts like an American chestnut (The Connecticut Agricultural Experiment Station began their chestnut program at Brooklyn Botanic Garden, and it was reinstated in the 1980s).

Almost 20 years later, noted corn geneticist Dr. Charles Burnham made a then-radical suggestion: Instead of crossing hybrids back to the *Chinese* parent in hopes of further strengthening blight resistance, the second,

## Breeding for Resistance: TACF and the Burnham Hypothesis

by Anna Huckabee Smith

*Where does TACF’s breeding program stand today?*

third and fourth generations should be *backcrossed* to *American* trees, which would bring back the desired American characteristics. Blight resistance could be retained if each generation of trees was tested for resistance by deliberately infecting them with the blight and only carrying the breeding forward with those trees that showed significant resistance. The last backcross would be followed by an intercross generation; by breeding the survivors with each other, the genes for susceptibility to blight from the American parents could be eliminated. Eliminating the genes for susceptibility would make the first intercross trees true breeding for blight resistance and would increase their resistance. Repeat this intercross one more time, and you would end up where everyone wanted to be: with mostly American chestnuts where every tree has high levels of blight resistance. Burnham hypothesized that under careful breeding protocols it would take only three backcrosses and two intercrosses (six generations, or about 30 years) to accomplish this goal (see backcross chart page 12).

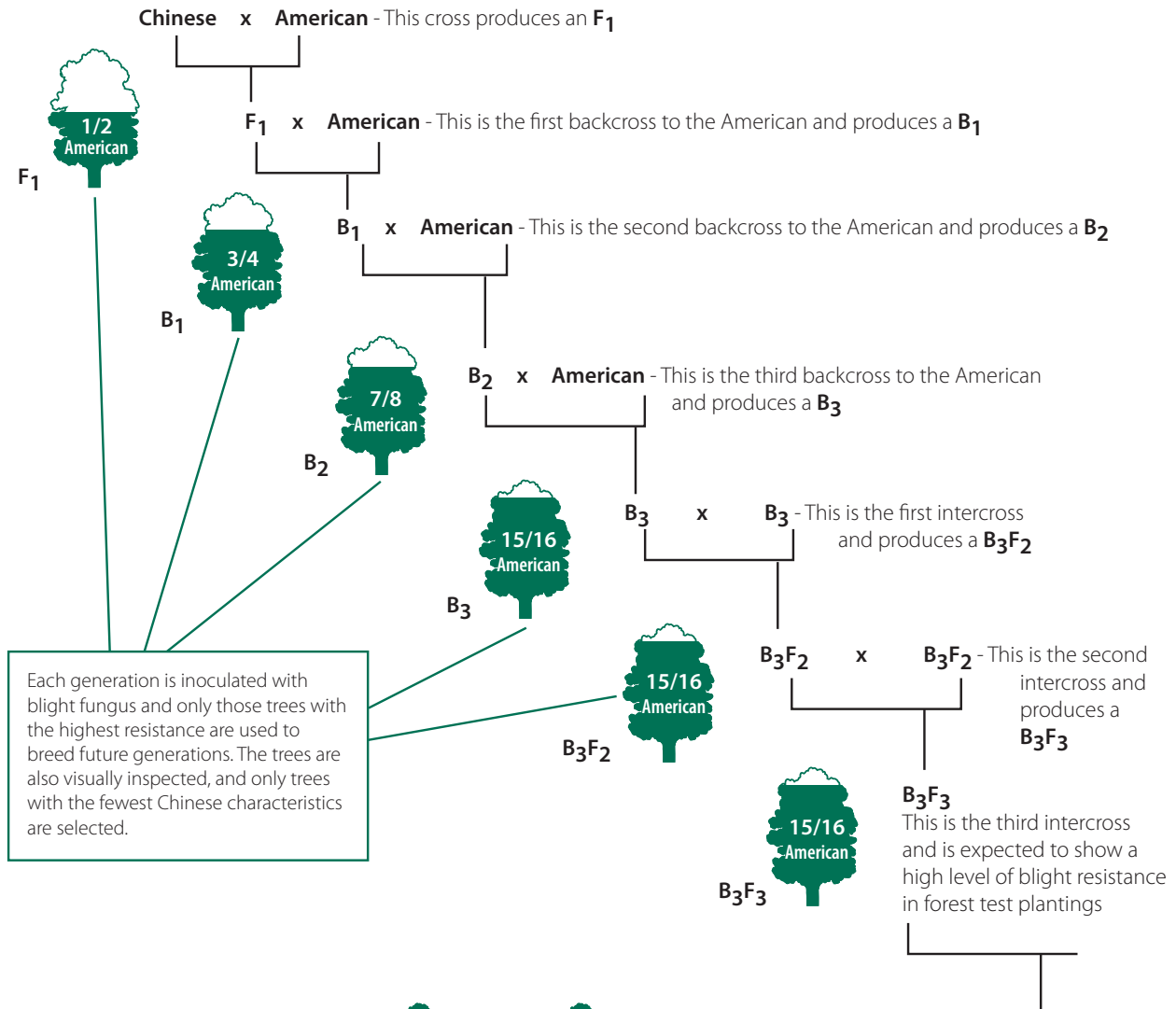
In 1983, Burnham joined forces with Philip Rutter, Dr. David French, Dr. Larry Inman, Don Willeke and others to found The American Chestnut Foundation as a non-profit organization tasked with evaluating what became known as the “Burnham Hypothesis.”

### **From “The Burnham Hypothesis” to “The Meadowview System”**

In 1989, the foundation established Meadowview Research Farms and hired Dr. Fred Hebard to head up

## THE AMERICAN CHESTNUT FOUNDATION'S BACKCROSS BREEDING PROGRAM

TACF's backcross breeding program begins by crossing an American chestnut and a Chinese chestnut. This is followed by three successive generations of crossing back to American chestnut trees to restore American characteristics. In between each breeding step, the trees are inoculated with blight fungus (*Cryphonectria parasitica*) and only those trees showing strong blight resistance and American characteristics are chosen to breed additional generations. For the final two generations, trees with proven blight resistance are intercrossed with each other to eliminate genes for susceptibility to blight introduced from the American parents.



### Breeding, testing and evaluation continues.

TACF's breeding program will continue to increase existing levels of blight resistance and integrate additional sources of blight resistance into the breeding populations. For more information on TACF's breeding and restoration programs call 1-(828)- 281-0047 or visit our website at [www.acf.org](http://www.acf.org).



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Volunteer Terry Stamper bags female flowers on a large American chestnut to prevent pollination from unwanted sources.

the breeding program. Hebard had studied chestnut trees extensively in his undergraduate, graduate and postdoctoral work, and all of his practical experience was needed at the start of what would become his life's work. "It was my dream job," says Hebard. "I already knew how to breed chestnut trees for blight resistance. Dr. Burnham had devised a crossing plan, and it was up to me to implement it."

One of the first tasks was to begin collecting American and Asian species to cross. Even after so much devastation, the American chestnut was not extinct in the wild. Millions of stump sprouts were, and still are, growing in the eastern forests, and because the trees can reproduce at an early age, some of these stump sprouts and a handful of larger surviving chestnut trees produced pollen and nuts that could be harvested. Scientists and volunteers collected the pollen and nuts to make crosses between the wild American trees and known Chinese cultivars, most prominently "Nanking," which had shown itself to have a high level of resistance. The researchers also got a boost by using material bred during earlier chestnut research by the USDA and The Connecticut Agricultural Experiment Station.

Then as now, the breeding method established at Meadowview Research Farms follows the Burnham system closely. First, a Chinese chestnut is crossed with an American chestnut tree (see diagram). The resulting progeny of this first cross is called the  $F_1$  generation. The next breeding cycle consists of backcrossing a select  $F_1$  to another American parent, resulting in the first backcross or  $B_1$  generation. Select progeny in the  $B_1$  generation are then backcrossed to yet another American parent to yield the  $B_2$  generation. A third cycle of backcrossing produces the  $B_3$  generation. It is important to introduce new American parents to the

gene pool at every backcross in order to minimize inbreeding and maximize genetic diversity.

At the end of this process, TACF has trees that are on average 15/16ths, or 94%, American. After each backcross, any trees that have visible Chinese characteristics are culled. The remaining trees look American and contain just 6% Chinese genes. But which trees have retained the most important Chinese characteristic - blight resistance?

To find out, the trees must undergo a blight-resistance test. In early June, when they are between two and five years of age, two small holes are bored through the tree's bark and a small disc of agar containing one of two strains of the blight fungus is inserted into each hole. The bottom hole receives the most virulent blight strain (Ep155) while the top hole receives a less virulent strain (SG2-3). After five months and again at eleven months, the cankers are inspected and their lengths measured or else they are visually graded based on size and symptoms. Those trees with the smallest cankers are chosen for breeding and all others are culled.

Because each backcross reintroduces the weak American genes for non-resistance into the genome, intercrosses are needed to weed them back out. At this point the surviving  $B_3$  trees have demonstrated some level of blight resistance. But in order to strengthen that characteristic, the  $B_3$ s are intercrossed with each other.



Dr. Fred Hebard working in the Glenn C. Price Lab at Meadowview Research Farms



To inoculate a tree, a small sample of a known strain of chestnut blight is removed from a petri dish of agar.



The blight sample is then placed into a hole that has been punched or drilled through the soft thin bark of a young chestnut tree. The hole is then covered with tape.

This creates a new generation called  $B_3-F_2$  that has a chance to inherit blight resistance from *both* of its parents. The two orchards that currently contain these fifth-generation trees are known as Legacy Tree orchards. The final step occurs when these trees open pollinate with each other, creating the  $B_3-F_3$  generation, which is currently TACF's most advanced seed (Restoration Chestnuts 1.0). Trees grown from these seeds are 94% American chestnut, morphologically similar to wild American chestnut trees, *and* contain significant levels of blight resistance.

### Developing Regional Adaptation and Genetic Diversity

TACF scientists have long believed that regionally adapted Restoration Chestnut trees would have a higher survival rate than those that came from a distant geographic area. American chestnut trees, although genetically similar throughout their range, may have unidentified micro-adaptations to local conditions such as soil moisture, temperature, elevation, timing of bud break, and day length. In short, a tree bred in Maine from trees that have thousands of years of history adapting to the conditions in Maine may survive better in Maine than a tree from Georgia, and vice-versa. These regional breeding orchards start with mating of advanced trees from Meadowview ( $B_2s$  for example) with trees from the region where the orchard will be planted. The breeding program is thus completed using local parents that add both regional adaptations that may help the trees survive and much needed genetic diversity.

Genetic diversity is another essential element required to establish a healthy population of wild trees in a forest environment. The goal of the breeding program is to enable the American chestnut to resume evolving by itself, as a wild species. Chestnut, like many forest trees, has a high degree of genetic diversity, meaning that no two trees are exactly alike. One individual might contain genes that help the tree adapt better to limestone soils while another tree might contain genes that enable it to withstand late spring frosts. Those genes may not be prevalent in all individuals, but their presence in the population of trees may have helped the species adapt to environments as they changed over time. Additionally, a base population of trees is needed to avoid inbreeding, which can lead to collapse of local populations. At each location where breeding occurs, be it Meadowview or a state chapter, twenty different individual lines of trees are bred to each source of blight resistance, to maintain a viable base population. The aggregate of populations is large enough that mutation can offset long-term erosion of genetic diversity by genetic drift. Genetic drift is the gradual loss of genes due to random fluctuations in their frequency; if a population is too small, there are not enough new genes created by mutation to offset drift and maintain diversity.

TACF's regional breeding program, run by its state chapters, adds both genetic diversity and local adaptation. TACF began the state chapter system in the 1980s to implement regional planting of breeding orchards. Today, TACF's 16 state chapters have established more than 300 breeding orchards in 21 states. These orchards





Harvested pollen is sifted through a fine mesh to separate the pollen-laden anthers from filaments and debris.


are fueled by Meadowview's "Mother Tree" and "Father Tree" programs. In the Father Tree program, pollen from wild American chestnut trees from a specific region is brought to Meadowview to pollinate advanced trees. In the Mother Tree program, pollen from advanced trees is taken from Meadowview to pollinate wild American chestnuts in specific regions. The progeny from these pollinations are then planted in the breeding orchards in the region from which the "Mother Tree" or "Father Tree" originated.

### Looking to the Future

Since 2005, TACF has harvested increasing numbers of seeds every year from the Legacy Trees. Seeds of this B<sub>3</sub>-F<sub>3</sub> generation are called Restoration Chestnuts 1.0. The 1.0 signifies that they are just the first in a series of potentially blight-resistant trees. In 2009, the first of these Restoration Chestnuts 1.0 were planted in real forest environments. While this reforestation is only at a test phase, it represents a fundamental milestone for TACF.

TACF continues to plant and develop regional breeding orchards. Each of the states in the American chestnut's native range will eventually develop its own Legacy Tree orchards and the seeds from these orchards will steadily increase the amount of chestnut reintroduction. The long-term stability of the blight resistance being bred into American chestnut by TACF is a special point of concern and subject of research. Currently, only three sources of blight resistance are used widely in the program, and, like many other plant pathogens before it, the blight fungus might evolve to overcome current sources of resistance. A major objective is to increase the number of sources of resistance being bred into advanced

material. The plan is for the Meadowview Research Farms to develop new sources of resistance that will be added to advanced material that will eventually be sent to state chapters that will complete the breeding process.

Today, the breeding program continues at Meadowview, and has steadily grown. Meadowview Research Farms currently covers 160 acres, growing more than 55,000 trees in various stages of crossing. Culling trees with Chinese morphology or insufficient resistance still occurs at even the Restoration Chestnut 1.0 level. Generations well past the sixth will be created as improvements are made and scientific advances in genetics help unravel the mysteries of the fungus, the virus that attacks the fungus, and the trees themselves. As Hebard states: "The ultimate proof of Burnham's Hypothesis and success will be long-term survival of large trees in the forest as a viable, self-sustaining population across their former range." 

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Meadowview Farms Director of Operations Jeff Donahue examines chestnuts being grown in containers in the greenhouse at the Glenn C. Price Laboratory.



In a poor environment, a resistant chestnut tree, like this B<sub>2</sub>F<sub>3</sub> tree, may be severely attacked by blight. The main stem on this tree has died and only sprouts remain.

Photo by Paul Sisco



In a good environment, a pure American chestnut tree with little blight resistance can grow long enough to produce nuts.

## The Effects of Environment and Time on Blight Resistance

by Dr. Paul Sisco

Chestnut trees are affected by both genes and environment—nature and nurture—and this is especially true of a chestnut tree’s response to attack by the chestnut blight pathogen. Healthy trees in a good environment will usually be more resistant to blight infection than trees under environmental stress. Stress can be caused by a number of factors such as drought, poor soil conditions, or damage by other pests and pathogens. Jones, Griffin and Elkins (1980) found that blight cankers on Chinese chestnut trees in the eastern United States were more numerous when the trees were at higher altitudes, in colder environments, in frost pockets, or where they were exposed to strong winter winds. In China, blight severity has been observed to increase on older trees and on trees in the northern part of the country (Zhou et al., 1993).

TACF does not expect its Restoration Chestnut trees to be blight-free in all environments and over the entire course of their lives. For one thing, *resistance* is not the same as *immunity*. An *immune* tree would never show blight symptoms. A *resistant* tree will most likely show at least some blight symptoms over its life span. And in a poor environment, even a resistant tree may be severely damaged by blight.

Younger chestnut trees with tight bark tend to be more resistant to blight infection than older trees with furrowed bark. The chestnut blight fungus needs an opening in the bark to cause infection, which is why initial cankering often occurs at branch points of the tree where the bark is split.

TACF scientists have worked over 20 years to develop American chestnut trees with blight resistance. But they will not know for sure

## How to interpret the codes of your Restoration Chestnut trees

Example: If your tree has the code W1-20-6, it means:

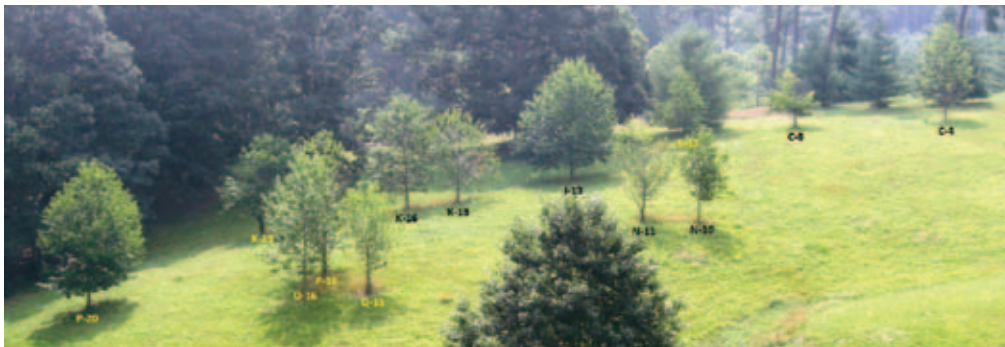
1. **W** It comes from the Wagner (W) farm – meaning it has the Graves source of resistance [“D” = Duncan Farm, Clapper source]
2. **1** It is from Replication 1 of 9 replications of a particular Graves “Line”
3. **20** It is from Graves Line #20
4. **6** It is from Tree 6 in the (Graves Line 20 / Replication 1) subplot

whether the resistance of a particular parent tree is adequate until the offspring of that parent have been tested for many years in a wide variety of environments.

This is where TACF members and cooperators receiving Restoration Chestnuts can really help by (1) keeping up with the numbering system on the labels (see chart above) and (2) reporting back on the condition of their trees over time. As data are collected, certain parents will be found to be better than others, because their offspring will have proved to be more resistant over time and in varying environments. 🌿



Cankers are growing on this pure American chestnut tree, especially at branch points where there is a break in the bark.



TACF needs its members to report back data over a number of years. At the Biltmore Estate in Asheville, NC, 18 trees remain of 276 planted at TACF's Annual Meeting in 1997. These trees have endured attacks by chestnut blight, drought, *Phytophthora* root rot, and gall wasp, yet several still look healthy and thriving.

Photo by Paul Sisco

### References:

Jones, C., G.J. Griffin, and J.R. Elkins. 1980. Association of climatic stress with blight on Chinese chestnut in the Eastern United States. *Plant Disease* 64:1001-1004

Zhou, E., K. Wang, and J. Lu. 1993. The conditions governing the occurrence of chestnut blight in eleven provinces of East China. *J. Nanjing Agric. Univ.*, English abstract.

# Determining Blight Resistance in Chestnut Trees

by Dr. Fred Hebard

When it comes to evaluating the level of blight resistance of the chestnut trees in TACF's breeding program, assigning accurate, consistent and meaningful levels of resistance is a combination of art and science.

The process of determining which trees have a high level of blight resistance begins in early June when we inoculate the trees with the blight fungus (*Cryphonectria parasitica*). Over the next several months the fungus will enter and spread through the tree's bark and wood (see "Introduction to Chestnut Blight," page 9). The tree attempts to fend off this attacker by walling off the fungus. The infected, dead bark tissue is known as a canker.

Chestnut trees react differently to inoculation. On trees with little or no resistance to blight, large cankers develop around the area where the fungus was introduced. Once a canker encircles a stem, the portions above die if the canker extends all the way to the vascular cambium. In contrast, trees with strong resistance may show little more than a small blister around the inoculation site.

Determining the size of a canker and associated symptoms forms the basis for assessing a tree's resistance to blight. We do this in late fall, November and December. Supplemental ratings might be done again in June, a year after inoculation.

Formerly, when the resistance of only a few hundred or thousand trees needed to be determined, we used a ruler to measure canker dimensions. Now that we determine resistance in tens of thousands of trees per year, we use a fast, visual, qualitative system of assessing canker size and severity.



Image 1: Small cankers show little sporulation and no canker expansion beyond that which occurs within two weeks of inoculation.

Cankers are placed in three classes: small, medium and large. These basic classes allow TACF to assign numerical rankings to cankers of 1, 2 or 3, corresponding to the small, medium and large classes, respectively. The numerical rankings also serve as rankings of the blight resistance of the tree. Ranking into the three classes is facilitated by obvious qualitative differences in canker appearance, as described in Images 1, 2 and 3. Although large cankers, especially those incited by Ep155, can show size differences between cankers given a rating of 3, there are no qualitative differences in appearance, so we do not attempt to distinguish them.

At times it is practical to utilize a more detailed qualitative rating. This is achieved by inoculating the trees with two different strains of the blight fungus: one using the highly pathogenic strain, Ep155, and the other using the weakly pathogenic, but still virulent strain, SG2-3. We then rate both cankers and create a composite ranking from 1-5 (see chart below) that is the sum of the rankings for the two cankers, minus one point. Using both strains enables us to measure resistance over a broader range than would be possible using just one strain. SG2-3 cankers enable us to distinguish trees with low to intermediate levels of blight resistance,




Image 2: Medium-sized cankers show some expansion beyond the flush that occurs within two weeks of inoculation, but generally are less than 5-10 cm in length, are not sunken and do not show abundant orange stromata (the erumpent pustules containing the fruiting bodies of the chestnut blight fungus).

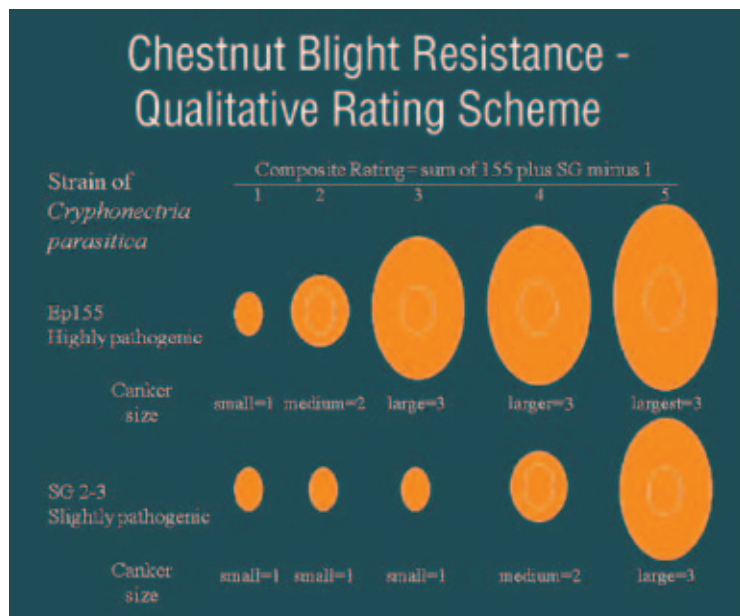


Image 3: On smooth-barked trees, large cankers generally exceed 10 cm in length and appear sunken with abundant stromata.

while Ep155 cankers enable us to distinguish trees with intermediate to high levels of resistance.

Using this system, pure Chinese chestnut trees commonly receive a composite ranking of 1 or 2 and pure American chestnut a ranking of 4 or 5. Their F1 hybrids are intermediate in blight resistance between the two parents and usually receive a rating of 3. The most blight-resistant straight backcross progeny usually receive a rating of 3, while the most blight-resistant backcross F2s usually receive a rating of 1.

Environmental variation from year to year or site to site can shift ratings about one level up or down. SG2-3 cankers also might respond differently to environmental variation than Ep155 cankers. Thus we include check trees of pure species and their F1 hybrid in most plantings where we intend to evaluate trees for blight resistance. 



This chart illustrates the qualitative system for rating disease severity that allows screening many thousands of trees per year for blight resistance.

The individual severity scores from inoculation with two strains of the blight fungus are added together, and one point is subtracted to create the composite score. For example, a medium-sized canker incited by blight strain Ep155 has a score of two and a small canker incited by strain SG2-3 has a score of one. Adding the Ep155 canker's score of two to the SG2-3 canker's score of one and then subtracting one gives a composite score of two.

# Blight Resistance: It's in the DNA

by Rebecca Hirsch

Photos courtesy of SUNY-ESF

Why do American chestnut trees die from a blight caused by the fungus *Cryphonectria parasitica* while Chinese chestnuts so often fight it off? Scientists are looking for answers to that question in the chestnut's DNA. In work supported by TACF and the Forest Health Initiative, teams of researchers from TACF, the US Forest Service, Pennsylvania State University, University of Georgia, Clemson University, and State University of New York College of Environmental Science and Forestry (SUNY-ESF) are working to map and sequence the chestnut's genome and identify the genes that contribute to blight resistance. What they learn may aid in the effort to restore the American chestnut to the forest ecosystem.

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Scientists have now pieced together detailed genetic maps of the Chinese chestnut cultivars 'Vanuxem,' 'Nanking,' and 'Mahogany,' and have approximately located genes for blight resistance in three regions on the 'Mahogany' map.

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## On the Trail of Resistance

A chestnut tree's genome—its complete set of genes—is housed in an ensemble of 12 chromosomes that together hold the instructions to make and operate the tree. Unravel one of the chromosomes and you will



Shoots grow from a transgenic somatic embryo. These are multiplied again, rooted, and finally, after many stages, they regenerate into a new plant.

find long strands of DNA made of four repeating units called bases. The bases come in pairs: one strand of bases paired with another strand, forming a double helix. It is the order of the bases that determines the meaning of the genetic instructions, and physical differences between the Chinese and American chestnut trees—in traits like height, leaf shape, and the ability to fight off pathogens—can be traced back to differences in the DNA.

Studying the genome of an organism is no small task. The chestnut's genome is immense. The best estimate is that it contains, give or take, 800 million base pairs.

Genetic mapping is a way for scientists to negotiate this large landscape. Researchers identify markers that act as mileposts along the chromosomes. The markers can be snippets of DNA or unique sequences of bases. Putting the markers together to create a map of the genome serves a number of useful purposes. A map gives researchers a way to compare chestnut to related species like beeches, oaks, and other forest trees. It serves as a jumping off point for sequencing the chestnut's genome, determining the exact order of those 800 million or so base pairs. And it can reveal the location of genes that control certain traits such as blight resistance.



SUNY-ESF Technician Kristen Russell transfers somatic embryo clusters onto fresh medium.

Scientists have now pieced together detailed genetic maps of the Chinese chestnut cultivars ‘Vanuxem,’ ‘Nanking,’ and ‘Mahogany,’ and have approximately located genes for blight resistance in three regions on the ‘Mahogany’ map. Additional genes for resistance may be identified in further research. These three regions, called loci, are spots where genes for blight resistance reside. Researchers are now zeroing in on the three loci, sequencing the DNA in each region in an effort to find the specific genes that contribute to blight resistance. The loci contain hundreds of genes—the large majority of which have nothing do with resistance—which means that scientists must use detective work to narrow the search.

One clue researchers look for in finding a gene for blight resistance is evidence that the gene is turned on in blight cankers. Scientists have screened cankers in American and Chinese chestnut trees to determine which genes are active. They are particularly interested in genes that are turned on at high levels in the Chinese tree but are present only at low levels in the American tree when challenged with the blight. Such a pattern makes a gene a candidate for blight resistance.

Another clue researchers look for is genes that are similar to disease resistance genes from other plants. Scientists studying the blight resistance loci have noticed similarities to loci in peach that contain genes for disease resistance. The peach genes help fight powdery mildew, another fungal disease. Such similarities can greatly aid in identifying the genes that encode resistance in chestnut.

### Testing Resistance

Once researchers have identified likely candidate genes for blight resistance, they can perform a direct and powerful test of each gene’s function by adding the gene to an American chestnut tree and testing whether the added gene offers the tree any additional resistance to the blight. This approach allows researchers to directly address the question: Does this gene confer resistance to the blight?

To carry out this test, the gene is added to a soil bacterium known as *Agrobacterium* (*Agrobacterium tumefaciens*). *Agrobacterium* has the singular ability to attach itself to a plant and inject a small piece of DNA into a plant cell. “You can think of *Agrobacterium* as a little shuttle,” says Dr. Joe Nairn, whose lab at the University of Georgia is involved in this effort.

“You put the gene in the shuttle, and the shuttle delivers it to the plant cells.” *Agrobacterium* containing the gene of interest is mixed with American chestnut embryos, and the end result is that the injected DNA ends up spliced into the plant’s own DNA, a process known as genetic transformation.

Next the transformed embryos are moved to a growth medium—a liquid or gel-like substance filled with nutrients and hormones—and the embryos are grown into new plants. The transgenic trees are then moved to pots and later to a test-plot outdoors, where they can be tested against control plants to determine how well they can resist the blight.

Dr. Scott Merkle, a chestnut researcher at the University of Georgia, stresses that safety is a focus in working with genetically engineered trees. Growers follow strict rules handed down by the USDA and other regulatory agencies. Nurseries are fenced, gated, locked. Inspectors visit regularly. Flowers are clipped off or bagged to prevent the spread of pollen. Every tree is labeled and monitored, and even pruned branches are tracked and discarded safely. “The major concern,” says Dr. Merkle,



Isolating embryos from seeds is the first step in growing them in tissue culture. They will multiply into thousands of new embryos, called somatic embryos, before being transformed with new genes.

“is that we don’t allow any of the genes that we’re testing to move into the wild population.”

### **Solutions from the DNA**

It will likely take years for researchers to tease apart the genetic pathways that enable Chinese chestnut to fight off the blight. Dr. Paul Sisco, retired staff geneticist with TACF, cautions that the system that emerges may be complex. He envisions a scenario in which different genes might control resistance in different Chinese chestnut cultivars or in different Asian species such as Japanese chestnut. Researchers are already getting glimpses of this complexity, with evidence that some of the three blight-resistant loci identified in Chinese chestnut cv. ‘Mahogany’ may respond differently depending on the particular strain of *C. parasitica*.

Even though it will likely take years for scientists to unravel blight resistance, DNA studies could soon show direct benefits to the effort to restore the American chestnut. Right now trees in the breeding program must be grown for two to four years or more before researchers can determine their level of blight resistance. According to Dr. Sisco, the use of genetic markers that lie near the resistance genes could make the backcross breeding program more efficient. “Using DNA markers to identify resistance in newly emerged seedlings could save us a lot of time, space, effort, and money,” says Sisco.

Some researchers envision using genetic engineering to produce a blight-resistant American chestnut. They are experimenting with adding genes to the American chestnut in the hopes of creating a transgenic tree that can resist the blight. They are also experimenting with

adding resistance genes from other species. Leading in this effort are Drs. Bill Powell and Chuck Maynard at SUNY-ESF and Drs. Merkle and Nairn at the University of Georgia.

One of the most promising projects for Powell and Maynard involves transforming American chestnut with the oxalate oxidase gene from wheat. The gene encodes an enzyme that breaks down oxalic acid, a chemical present in large amounts in blight cankers and toxic to chestnut tissues. Their hypothesis is that the enzyme will neutralize the acid, prevent the canker from growing, and enhance the tree’s resistance. The first of these transgenic trees were planted in early 2011.

Merkle and Nairn believe that genetic engineering could also be used to create a chestnut that can resist ink disease caused by *Phytophthora cinnamomi*. This deadly pathogen, once confined to the southeastern part of the chestnut’s range, may move farther north and to higher elevations should the earth warm. “It’s worse than chestnut blight because there’s no resprouting from the stumps,” says Merkle, “Once a tree gets *Phytophthora*, it’s dead and it’s not coming back.”

Researchers caution that transgenic trees would probably not be used directly for reforestation, but might be crossed to surviving American chestnut trees as a way to build in genetic diversity. Yet whether the public will welcome genetic engineering as a way to save the chestnut remains to be seen. “It’s a whole other question,” says Nairn. “There’s a large community that will have to address that.” 🌿