

Research Objectives of the American Chestnut Foundation, 2004-2014

The goal of The American Chestnut Foundation (TACF) is to restore *Castanea dentata* (Marsh.) Borkh. as a dominant tree to the forests of eastern North America. The primary method by which we are trying to accomplish this restoration is to backcross the blight resistance of the Chinese chestnut, *Castanea mollissima* Blume, into the American chestnut and to plant blight-resistant trees from that program into the forests of the Appalachian Mountains. However, we support all methods by which restoration might be accomplished.

We wish to restore the American chestnut because it is threatened with extinction due to chestnut blight, incited by *Cryphonectria parasitica* (Murr.) Barr. American chestnut used to be a major forest tree, of importance to people and the environment. Chestnut's former economic importance may facilitate reintroduction of blight-resistant trees that we have bred, by providing an economic incentive for their planting.

Breeding and restoring a dominant forest tree over such a wide area may lead to increased understanding of the genetic and physiological bases for that dominance. We plan to examine these bases where possible, as well as the genetic and physiological bases of more specific traits, such as blight resistance.

TACF operates a central research facility in Meadowview, Virginia. In addition, it coordinates a network of state chapters staffed by volunteers who engage in breeding and other research to restore the American chestnut. Finally, it funds and supports extra mural research at cooperating institutions, such as universities, in furtherance of its objectives. This document presents and discusses the research objectives of the foundation over the next 10 years, and discusses research objectives for the following 20 years. These are arranged in order of priority for the central facility in Meadowview, for the state chapters, and for further breeding and testing of blight-resistant trees in eastern forests.

OBJECTIVES

A: Meadowview

- 1) Complete planting of two seedling seed orchards and selection of trees reasonably true breeding for blight resistance. One seed orchard will be for the 'Clapper' source of blight resistance, and one for the 'Graves' source.
- 2) Advance a third source of resistance derived from the Nanking cultivar of Chinese chestnut to third backcross in 20 lines of American chestnut.

- 3) Determine what additional sources of blight resistance might be useful in restoring American chestnut and obtain first or second backcross F_2 s homozygous for the genes conditioning blight resistance.
- 4) Continue breeding of large, surviving American chestnut trees that have shown low levels of heritable blight resistance, to determine whether that blight resistance might be increased to a usable level.
- 5) Evaluate traits of chestnut that might be related to its ability to grow as a dominant forest tree.
- 6) Begin advancing one source of blight resistance to sixth backcross, in anticipation of comparing its field performance to that of third backcross trees.

B: State Chapters

- 1) Finish advancing the 'Clapper' and 'Graves' sources of resistance to third and fourth backcross, by crossing 20 separate pollens for each onto 20 separate American chestnut trees from each of NC, ME, MA & PA, and complete planting of two seed orchards in each of those states. Selection of trees homozygous for the genes conferring blight resistance would be completed in the following 10 years.
- 2) Complete advancing one source of blight resistance as above in KY ('Graves'), TN ('Clapper'), MD ('Clapper'), IN ('Clapper'), and VT ('Graves'), and complete planting one seed orchard. Selection of trees homozygous for the genes conferring blight resistance would occur in the following 10 years.
- 3) Form new state chapter centered near northern WV or eastern Ohio and initiate backcrossing, and likewise in VT and AL. Revitalize chapter in CT and initiate backcrossing. These four chapters would more-or-less complete the infrastructure for our regional breeding program.
- 4) Continue supporting research at Syracuse University and the University of Georgia aimed at transforming chestnut with DNA plasmids containing genes for blight resistance (NY Chapter and national).
- 5) Initiate backcrossing onto 20 American chestnut trees of additional sources of blight resistance obtained under Objective 3 in Meadowview, with each chapter using a separate source of blight resistance.

C: Testing & Further Breeding

- 1) Organize a symposium to discuss results from cooperators on establishing American chestnut in the forest, with a view to formulating planting guidelines.
- 2) Initiate testing in the forest of trees obtained from Meadowview seed orchards. Supplement with trees from chapter seed orchards where feasible and appropriate.
- 3) Initiate testing in orchard settings (rather than forest) of trees obtained from Meadowview seed orchards, with a view to continuing improvement of the breeding population and creation of B_3 - F_3 seedling seed orchards. Improvement would be achieved by both family-level selection and selection of individuals within families. Repeat with trees from chapter seed orchards as these come into full production from all breeding lines.
- 4) Initiate a longitudinal demographic and epidemiological survey of American chestnut sprout populations in areas likely to be undisturbed for the foreseeable future, such as National Parks and National Forest Wilderness Areas.
- 5) Initiate provenance tests (common garden studies) of chestnut from our regional seed orchards.
- 6) Initiate testing of blight-resistant backcross trees in the presence of hypovirulent strains of the blight fungus, to assess whether combining the two control methods gives better remission of disease than either alone.
- 7) Initiate wide scale planting and monitoring of blight-resistant American chestnut in the Appalachian Mountains with a goal of planting 200,000 acres over the next 30 years.

MATERIALS AND METHODS

It may be helpful in following the discussion to describe the basic backcrossing method of plant breeding we have adapted to breeding American chestnut trees for resistance to blight. We first cross the two species to obtain a tree that is one-half American, one-half Chinese chestnut. This first hybrid, or F_1 , is then backcrossed to American chestnut to obtain trees that are three-fourths American, one-fourth Chinese, on average. First backcrosses (denoted B_1) which test as resistant to blight are then backcrossed again to American chestnut, to obtain trees which average seven-eighths American, one-eighth Chinese, denoted B_2 . A third cycle of selecting and backcrossing produces trees which average fifteen-sixteenths American, one-sixteenth Chinese, denoted B_3 . A final step is to intercross third backcrosses with each other to produce trees, denoted B_3 - F_2 , which have a chance of inheriting the genes for blight resistance from both parents; those with

progeny with no genes for susceptibility to blight would breed true for those genes and serve as the mother trees to produce nuts for reforestation.

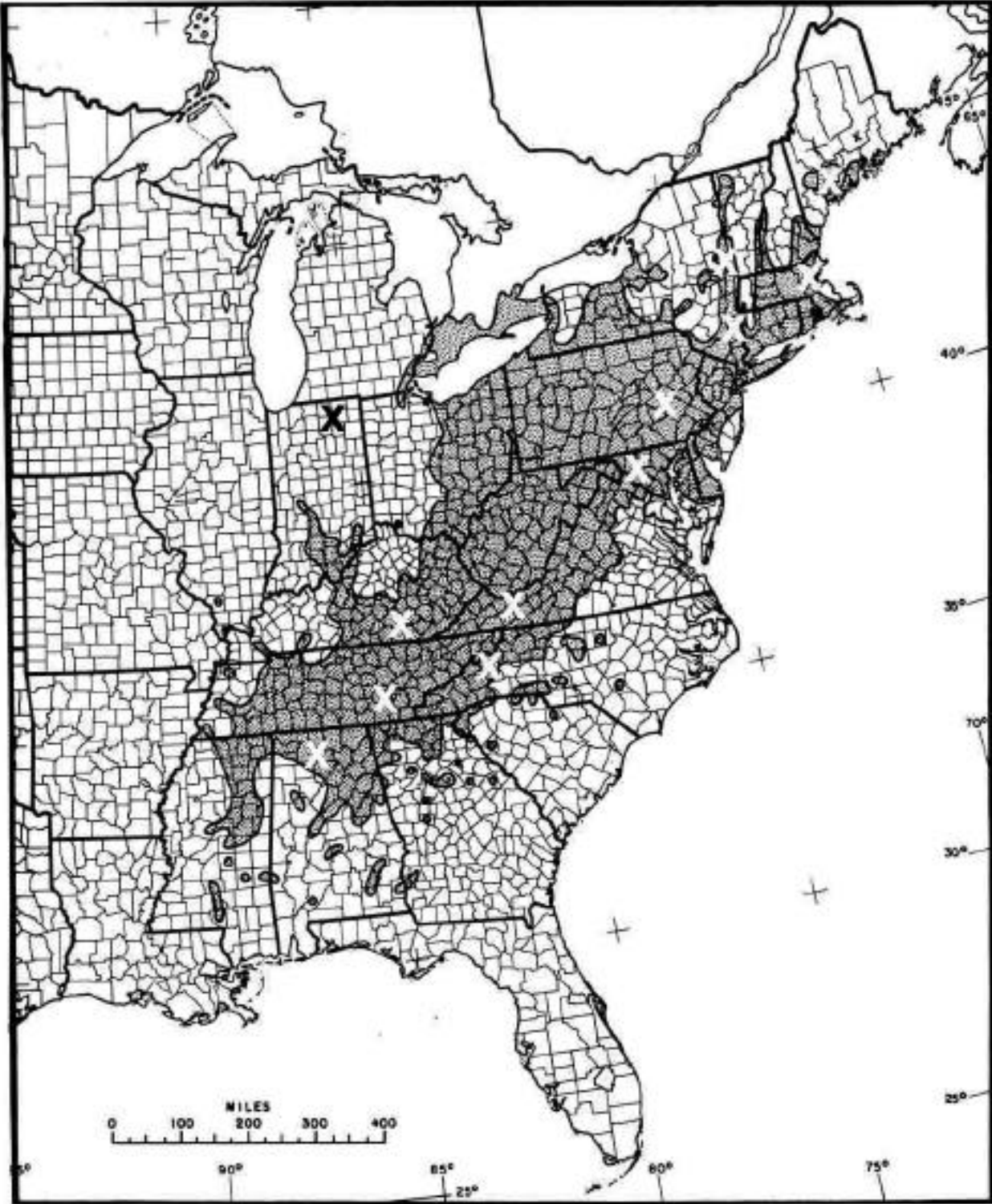
To avoid inbreeding, we use a different American chestnut at each step of backcrossing. We also use 20 separate sets of American chestnut trees for the last two steps of backcrossing to avoid inbreeding during the intercross generations. These sets are being replicated at about ten locations around the country for three sources of blight resistance (and it is possible that four more sites will be added). The American chestnut parents also differ between sources of blight resistance. This will both provide adaptation to local environments and increase the number of direct American parents to 600 (20 sets x 3 sources x 10 sites), thus capturing most of the alleles and helping ensure a viable population, in accord with the famous 50/500 rule of ecological restoration (Franklin, 1980). Namkoong (1991) estimated that “A few thousand samples are needed to save most alleles in most populations.” Counting the American chestnut grandparents of these crosses, we are getting close to that number.

Figure 1 depicts the locations of our state chapters in the current range of the American chestnut. We have most of the range fairly well covered except for the region around northern West Virginia, western Pennsylvania and eastern Ohio. We hope to establish another chapter in that region, as outlined in Objective B.3. The map also shows breeding locations in AL, CT and VT that are not as advanced as the other sites.

Ellingboe (1994) and Hebard (1994a) have discussed the theory and practice, respectively, of the breeding program and the initial crossing steps. Hebard has presented designs for seed orchards and methods for producing seed in them (2002) and methods for introducing additional sources of blight resistance into our chapter breeding programs (2001). Rutter (1991) described the crossing technique being employed, Hebard (1991) discussed locating flowering American chestnut trees, and Hebard and Rutter (1991) outlined planting methods suitable for orchards. Anagnostakis (1982) developed methods for crossing the blight fungus.

Details of testing of trees in the forest are presented in the report from our Testing Task Force, which is attached.

Kubisiak, *et al.*, (1997) have presented methods & results for mapping with molecular markers in chestnut and for QTL analysis of blight resistance and other traits. Hebard (1994b) presented methods and results for analyzing selected morphological markers. In general forest plantings under Objective 7, each planting would consist of blocks of trees from the same source of blight resistance to ensure that most progeny had a



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Figure 1.—Natural range of American chestnut.

Figure 1, Range map of the American chestnut and approximate locations of TACF chestnut breeding stations, marked by a white "x" in all states but Indiana, which is marked by a black "x."

reasonable chance of being homozygous for genes for blight resistance. Blocks from separate sources of blight resistance would be planted adjacent to each other where possible to allow reassortment and recombination of resistance genes between sources at the border between blocks, and to increase the overall genetic diversity. Regionally adapted seed would be used predominantly (>90%), with some addition of seed from other regions to increase overall genetic diversity. The seed from other regions could be planted in separate blocks or mixed, as appropriate to the planting and to further experience.

The most difficult aspects of this program may center around maintaining organizational continuity among the various program elements over the course of the project. TACF has managed to do this up to the present. Careful, open planning such as documented in this proposal will help greatly in maintaining organizational continuity into the future.

DISCUSSION

The need for trying to restore the American chestnut tree to our forests is almost self evident. It is estimated that mast production in the forest declined substantially with loss of the chestnut (Diamond, *et al.*, 2000). Chestnut was a much faster growing tree than oak species and other replacement species (Ashe 1912, Zon 1904); restoring it would increase rates of timber growth on chestnut sites by up to 100 percent. Most important perhaps, is that we should not sit idly by while trees slide into extinction.

The key question in our proposed restoration plan is whether or not it will work. This question will be addressed below from a number of perspectives.

Blight resistance. The most important factor in our success is ensuring that the products of our breeding program have levels of blight resistance similar to that found in Chinese chestnut, which is the most blight-resistant species of chestnut (Berry, 1960, and citations therein). Trees with lesser levels of blight resistance become too disfigured by blight to thrive in the forest.

As part of the normal sequence of breeding, we screen our trees for blight resistance at each generation. Thus far, all generations have had the expected levels of blight resistance. Because blight resistance is incompletely dominant, backcross trees, which have an American chestnut parent, are at best intermediate in resistance between Chinese and American chestnut, since they inherit genes for susceptibility to blight from their American parent. We only see high levels of blight resistance, such as occurs in Chinese chestnut, when we intercross two backcross trees with each other, rather than crossing them with an American chestnut. Intercrossing opens the possibility that the progeny will inherit only genes for resistance to blight from their parents and no genes

for susceptibility to blight. To date, we have tested progeny from intercrosses of the F_1 , B_1 and B_2 generations, to produce F_2 , B_1-F_2 , and B_2-F_2 progeny, respectively. We have recovered highly blight-resistant individuals from among the progeny of each type of cross.

This recovery of highly blight-resistant progeny is illustrated in Table 1 for F_2 s and B_1-F_2 F_2 s from controlled pollinations that were tested for blight resistance in 1993 and in Table 2 for two sets of B_2-F_2 progeny from controlled pollinations that were tested for blight resistance in 2003. We recovered two F_2 progeny in the smallest canker-size class, four B_1-F_2 progeny, three B_2-F_2 progeny from the 'Clapper' source of blight resistance and three from the 'Mahogany' source. (Discriminant analysis and survival additionally indicated that some individuals in the next two larger size classes also were highly resistant to blight). Figure 2 is a photograph of one of the highly blight-resistant B_1-F_2 progeny taken in 2004, 11 growing seasons after inoculation.

Table 1. Number of trees in canker size classes in 1993.

Cross Type	Size Class (cm)						
	1.0-2.6	2.6-4.2	4.2-5.8	5.8-7.4	7.4-9.0	9.0-10.6	10.6-
American					3	5	2
F_1 Nanking				2	4	3	
Seedling Chinese		2	7	3			
Meiling Chinese		1	2	2			
Nanking Chinese	3		2				
F_2 Mahogany	2	7	31	50	49	29	17
B_1-F_2 ClapperxGraves	4	25	84	116	112	54	4

Table 2. Number of trees in canker size classes in 2003.

Cross Type	Size Class (cm)						
	1.0-2.0	2.0-3.0	3.0-4.0	4.0-5.0	5.0-6.0	6.0-7.0	7.0-8.0
American			4	2	2	2	1
F_1 Nanking		1	2	3	1		
Chinese	3	3	3	6			
B_2-F_2 Clapper	3	11	15	37	16	12	3
B_2-F_2 Mahogany	3	11	21	31	14	14	1



Figure 2. Highly blight-resistant Chinese to American B_1-F_2 , 13 years old, 11 years after inoculation with *Cryphonectria parasitica*. The tree is to the left of and behind the dog.

Results of genetic mapping of blight resistance in the F₂ progeny listed in Table 1 were reported by Kubisiak, *et al.* (1996). Hebard, Sisco & Wood (2000) also tested the blight resistance of some F₃ progeny of highly blight-resistant F₂s and found that some cross combinations bred true for blight resistance: all their progeny were highly blight resistant. We will continue to test our progeny for blight resistance and will continue to evaluate the hypothesis that we can recover progeny with high levels of blight resistance, that breed true for resistance

American Type. It is an hypothesis that three backcrosses of a Chinese x American first hybrid to American chestnut will be sufficient to restore the American type to our progeny, albeit an hypothesis based on experience in the breeding of many different crop plants and farm animals. To accelerate the recovery of American type we follow standard practice in backcrossing by selecting for American traits at each step, from among the trees that have adequate levels of blight resistance.

Currently, we use morphological traits to select for American type. It also could be done, perhaps more effectively, using molecular genetic markers. However, we have not yet acquired the extensive resources needed to attempt this, and only have a few more years of selection in the two principle sources of resistance we have advanced to third backcross. Thus we have not made selection for American type with molecular markers an objective in this plan. However, if we have the opportunity (somebody willing to test our progeny at little cost) we will pursue this line of research.

There appear to be two distinct types of European chestnut (Villani, *et al.*, 1999), one adapted to xeric sites and the other to mesic sites. The two types differ in numerous physiological parameters, but can be distinguished by habit: the xeric type has spreading, horizontal branches and the mesic type has upright, vertical branches. We also have seen family-level differences of the same type in second backcrosses to American chestnut of Chinese-American hybrids. The family differences depend on the American parent of the second backcross trees. We are following this difference in our progeny and hope to relate it to their performance in forest settings. Under Objective A.5, we also follow other traits for ecological adaptation, such as the time of bud flush in the spring. We also are placing these traits on molecular genetic maps where possible.

We will know with certainty that our trees will thrive like the American chestnut of old only after they have done so. It will take 50-100 years for our trees to reach 100 feet in height and diameters in excess of 2-3 feet, if they can. It will only take about 20 years, on the other hand, to advance one source of blight resistance to the sixth backcross, which should be enough to restore the American type as much as is possible (Briggs & Allard, 1953). This we intend to do, as outlined in Objective A.6.

One might think that we could deploy trees at each step of backcrossing to help determine how many backcross generations are needed. However, it requires a lot of time and land to establish a seed orchard with 20 lines of American chestnut from one source of resistance (Hebard, Journal of TACF 16(1):7-18, 2002), which is the minimum number we estimate are needed to avoid inbreeding (Hebard, Journal of TACF 8(1):21-28, 1994). Although we could do it with fewer than 20 lines, those might be a breeding dead end, and this is a practical breeding program, first and foremost

We must balance the possibility that more backcross generations might be needed against other needs of the breeding program, primarily the possibility that one or more of our sources of blight resistance could break down.

Breakdown of resistance. Once highly blight-resistant, American-type chestnut are restored into the forest, it is possible that the blight fungus could evolve means of overcoming their resistance. The resistance is then said to have “broken down.” One encouraging sign that our blight resistance might not break down is that no Chinese chestnut trees have been found in the U.S. that are as susceptible to blight as American chestnut, despite widespread planting. All Chinese chestnut that have been examined have cankers typical of intermediate to high levels of resistance, and the highly resistant cultivars have retained that trait, being canker free for the most part (Headland, *et al.*, 1976, and Hebard and Griffin, unpublished observations). So their resistance has not broken down. However, blight resistance from Chinese chestnut might break down after it has been backcrossed into American chestnut. The purpose of a number of our objectives is to help avoid breakdown of resistance, as well as testing for its occurrence.

Our primary strategy for avoiding breakdown of blight resistance at this point is to use more than one Chinese chestnut tree as a source of resistance. In addition, we are evaluating the merits of Japanese and American chestnut as a source of blight resistance, the latter under Objective A.4. Since blight resistance is not known to have broken down yet, we cannot isolate races of the pathogen specific to the resistance they break, which precludes a lot of experimental approaches used in pathosystems where resistance has broken down.

To implement our strategy, each breeding location is advancing three sources of blight resistance, two from our most advanced sources, which they share in common, and one separate source for each chapter. These activities are encompassed in Objectives A.1, A.2, B.1, B.2 and B.5. Previously, these three sources were mentioned in connection to the number of American parents involved in the breeding program. Here, they are being mentioned in regard to the number of Asian parents. If we can develop breeding

populations from each, we will have used about 12 separate Asian chestnut trees as sources of blight resistance. But which Asian trees should be used, and how can we tell they have different genes for blight resistance? Such questions will be addressed under Objective A.3, which will now be discussed more fully.

If two Chinese chestnut trees had identical genes for blight resistance, then the genes for resistance in their progeny would be identical, assuming all were transferred to the progeny. Using both trees would not add any more genes for blight resistance to our breeding stock than using only one of the trees (although we would be increasing genetic diversity on the American side).

One can detect whether genes for blight resistance from different Asian chestnut trees are at different loci, both by classical methods and by molecular mapping of resistance loci. The classical method is to cross two Asian chestnuts and look for segregation for blight resistance in their progeny: if one or more parents were heterozygous for blight resistance at one or more loci, blight resistance would segregate in their direct progeny; if the parents were not heterozygous, it would take two generations to see segregation. Currently, we are examining the progeny of such crosses and getting ready to make the second-generation cross.

We also have tried to locate the genes for blight resistance on molecular maps for different sources of blight resistance (as quantitative trait loci, QTL). However, these mapping studies were inconclusive because our measurements of blight resistance were too inaccurate to map it in all but one population. In one instance that inaccuracy was due to cool, wet weather during the 1996 growing season, which kept the cankers on American chestnut small: they were not much larger than those on Chinese chestnut. Similar weather occurred in 2003, and this phenomenon can be seen by comparing relative canker sizes between Tables 1 and 2. In 1993, a more “normal” growing season, we could differentiate clearly between cankers on Chinese and American chestnut (Table 1). In contrast, canker sizes on Chinese and American chestnut overlapped in 2003 (Table 2). The years 2003 and 1996 had the highest and second highest yearly rainfall on record for this region, respectively.

Our molecular mapping also has been plagued with inaccuracies due to segregation distortion, which we have been resolving before attempting more mapping of blight resistance. As bad luck would have it, some of the segregation distortion is occurring on two linkage groups near where resistance maps, and it is unclear whether those two linkage groups are on the same or different chromosomes. This is a critical point as the quantitative-trait loci for blight resistance mapping to these two linkage groups may be arising from the same gene. We have been cross-referencing our map to a map of

European chestnut using simple-sequence-repeat markers, but do not have enough markers yet to resolve all questions. We hope to obtain enough shortly. We also have been preparing other mapping populations from intraspecific crosses, which should show much less distortion of segregation ratios, again helping us resolve these difficulties.

Research of a more long-term nature is being sponsored by our NY State Chapter at Syracuse University and the national TACF has been sponsoring similar work at the University of Georgia. This work is to develop methods for genetically transforming chestnut. Adequate transformation techniques have been developed, but regeneration of transformed plants is still not possible. Aside from possible direct benefits if a suitable gene for blight resistance could be cloned into chestnut, this work also may help further dissection of the chestnut genome, allowing studies of gene knockout and addition. We are pursuing this research under Objective C.4. The research could be expanded to include other studies of chestnut blight genetics if opportunities present.

In addition to work on the host side, we intend to examine the genetics of virulence in the pathogen as part of Objective A.3. We plan to look for segregation for virulence among progeny of the blight fungus on various highly blight-resistant cultivars of Chinese chestnut as well as on backcross F_3 progeny derived from backcross F_2 parents homozygous for blight resistance.

If we found segregation for virulence in the blight fungus, this would indicate the presence of a race structure in the pathogen, which likely would lead to breakdown of the resistance from one or more of our sources. The chances are not great of finding resistance-breaking races of the pathogen before blight-resistant chestnut trees are planted over a large area, since resistance has not broken yet on Chinese chestnut. Only when we had races of the fungus would we be able to detect easily alleles for blight resistance that had similar effects against avirulent races. There is one report of statistical interaction in canker size between strains of the blight fungus and cultivars of Chinese chestnut, but this interaction reflected non-linearity in canker size rather than the existence of resistance-breaking races of the pathogen (Huang, *et al.*, 1996).

Most plant pathologists believe that deploying resistant plants over a wide area for several years is the only firm indication of durable disease resistance (Hulbert *et al.*, 2001). It is the one method that can exert selection pressure on the pathogen severe enough to uncover almost all races capable of breaking the resistance. This is a principle reason we wish to plant over such a wide area under Objective C.7; this will be our ultimate, best test of resistance stability.

We do not envision that planting on such a scale will begin until approximately 2015, and acknowledge that it will require much effort and further improvements to planting techniques. The 200,000-acre figure is also an upper limit on the number of acres of trees needed to exert significant selection pressure on the blight fungus. We are specifically recommending that no more than 200,000 acres be planted to our trees over the next 50 to 100 years until they have shown they can be dominant forest trees. Specific plans for monitoring these plantings will be formulated 10 years from now.

Testing. We have supported or assisted research on planting chestnut in the forest at Penn State University, the University of Tennessee at Knoxville and at Chattanooga, Bent Creek Experimental Forest in Asheville, NC, at Purdue University, the University of Kentucky and at the University of Illinois in Carbondale. These experiments should be nearing completion by 2005-2007, when we hope to sponsor a symposium on this topic, as outlined under Objective C.1.

The extensive planting envisioned under Objective C.7 is designed, in part, as the ultimate test of the stability of our blight resistance. But there are plenty of things that could go wrong before our resistance breaks down. Foremost among these would be that the trees fail to grow as well as the American chestnut of old or that their blight resistance is insufficient. The two traits, forest competitiveness and blight resistance are intertwined. Chinese chestnut cannot compete well in our native forests against trees like yellow poplar; it gets overtopped and shaded out. However, while Chinese chestnut trees are being killed by competing trees, the severity of blight on them increases (Hebard, unpublished observations). Cankers, on the other hand, weaken trees. So, even if our trees could compete successfully in the forest when blight is absent, in the presence of blight, trees with insufficient resistance might be weakened to the point where they couldn't compete. With a slight deficiency of either forest competitiveness or blight resistance, our trees probably will fail. The tests under Objectives C.2, C.5 and C.6 are designed to help determine whether our trees have adequate forest competitiveness and blight resistance.

We wish to set up common garden tests of our B_3 - F_3 s from the seed orchards at our different chapters under Objective C.5. In a common garden test, the seed from each chapter would be planted together in one location, so that we can compare their performance. Several tests would be planted at different locations, such as one in MA, one in PA, one in VA, etc. The common garden tests may serve as a negative control, if trees bred in one region fare well in their own region but do not fare well in others. The common garden tests also will help determine the range of adaptability of chestnut and help in the choice of seed for distribution. Finally, they may help test the effectiveness of the breeding done by different chapters.

There are a series of viruses which attack the blight fungus, reducing its virulence to the point where it can no longer damage chestnut trees. Such strains of the blight fungus are termed hypovirulent. Apparently because of these hypoviruses, chestnut blight is no longer a devastating disease of European chestnut (Bissegger, *et al.*, 1997). Hypoviruses also can protect American chestnut from blight to the point where they survive for extended periods, although such trees are disfigured and weakened enough that they probably could not compete with other dominant tree species in the forest (MacDonald & Fulbright, 1991). It is unclear why hypoviruses are more effective in reducing disease severity in Europe than in North America. However, part of the reason may be that European chestnut is less susceptible to blight than American chestnut (Berry, 1960, and citations therein). It has been observed that a slight increase in blight resistance might be enough to help hypovirulence control blight in North America (Dierauf, *et al.*, 1997). We intend to test this hypothesis under Objective C.6. Part of our motivation is to have a supplemental method of disease control that might enable our trees to become dominant forest trees even if their blight resistance is insufficient.

Chestnut in nature. While we are breeding chestnut trees in orchards, natural populations of American chestnut and the blight fungus will continue to evolve and change on their own as this pathosystem comes into equilibrium from the shock caused by introduction of the blight fungus. Equilibrium may not be reached for several millennia.

Currently, most American chestnut persist as small sprouts in the forest, incapable of supporting a large population of the blight fungus. Blight is endemic on these small sprouts. When chestnut exceeds a certain size and density, blight becomes epidemic once again. Currently, these local epidemics occur following removal of the forest canopy by disturbances such as wind throw, cutting, or mortality due to gypsy moth.

Many of the American chestnut sprouts in undisturbed forest are 40-60 years old, and have been living since the original pandemic. The entire cohort of trees is growing slowly larger and, undisturbed, will reach a size capable of triggering an epidemic at some point in the future (Hebard, 1982). Once that happens, many of the sprout clusters will die, never to sprout again (Griffin, 1989).

However, hypoviruses increase in prevalence once the population of the chestnut blight fungus rebounds during an epidemic. Usually, one or two American chestnut trees in a clearcut survive blight longer than usual, with cankers characterized by swelling of the host tissue and lack of penetration of the blight fungus to the vascular cambium. These

swollen cankers can be due to hypoviruses or to low levels of blight resistance in the host or to both (Hebard, *et al.*, 1984).

Since the original stand of chestnut was largely salvaged for timber as blight swept through, some trees with low levels of blight resistance may be hidden within the sprout populations, unable to demonstrate their resistance because they have escaped blight thus far. We are not suggesting that there will be very many sprouts with blight resistance. Currently, there are about 30 known American chestnut trees that are large and have been surviving blight for a long time (Table 3). Furthermore, while testing the pathogenicity of isolates of the blight fungus, many hundreds of sprouts showed no detectable variation in canker size (Griffin, *et al.*, 1983).

Table 3.

Counties with one or more large American chestnut trees (over 38 cm in diameter at breast height) that have contracted blight but survived (only some of these have survived since the original pandemic, others started growing subsequently).

County	Number of Trees
?, CT	1
Macon, NC	1
Wilkes, NC	1
Conway, NH	1
Coshocton, OH	2
Stark, OH	3
York, PA	1
Amherst, VA	3
Fairfax, VA	2
Fauquier, VA	2
Floyd, VA	2
Madison, VA	1
Washington, VA	2
Doddridge, WV	4
Monongalia, WV	3
Monroe, WV	2

It should be instructive to monitor the natural equilibration of blight on American chestnut, if nothing more than to serve as a negative control while we reintroduce chestnut into portions of the forest, but also to test the hypothesis that blight is leading

to extinction of the American chestnut. We are suggesting that the Smoky Mountains and Blue Ridge National Parks could serve as undisturbed areas in which permanent study plots could be established under Objective C.4. Additional study plots could be established and monitored in wilderness areas within National Forests and state forest lands.

Further Breeding. Release of B_3-F_3 plants into the environment is not envisioned to be the last step of the breeding program. Rather it is the first release in an ongoing breeding program. The selection of B_3-F_2 parents of the first release probably will be imperfect. Some parents probably will not be homozygous for blight resistance at all loci. We will want to cull these. We could detect them by test crosses to American chestnut or by the performance of their B_3-F_3 progeny. We plan to evaluate the performance of B_3-F_3 progeny as outlined in Objective C.4, essentially switching the breeding method from backcrossing to recurrent selection. We would select both for blight resistance and American type in seedling seed orchards of B_3-F_3 trees, among both individuals and open-pollinated families. Then the B_3-F_3 seed orchard would replace the B_3-F_2 seed orchards.

It probably would be best if B_3-F_3 seed orchards were planted on new land rather than trying to interplant among existing B_3-F_2 seed orchards. Our current vision would be to put them on public lands under the management of state and federal forestry agencies. We would not want to start planting these until most trees in our B_3-F_2 seed orchards were in production, which will not occur before 2015. By then, the tests initiated under Objective C.2 as well as other plantings will have indicated whether our trees were worthy of further breeding and effort by public forestry agencies.

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Report of the *ad hoc* Task Force on Testing
to
Science Cabinet of The American Chestnut Foundation

DRAFT (June 28, 2002)

Task force members: Al Ellingboe, Sharon Friedman, Fred Hebard, Hugh Irwin, Paul Sisco, Scott Schlarbaum, Kim Steiner (chair)

Purpose of the task force (by our interpretation): To develop recommendations for testing the form, adaptability, botanical characteristics, and durability of resistance of chestnut progenies intended for release and deployment as 'blight-resistant American chestnuts.'

Assumptions:

- The goal of TACF is to bring blight resistance into wild, naturally regenerating populations of *Castanea dentata* in Appalachian forests and, by doing so, restore the species to its former role. Achieving this goal requires the use of non-native alleles because the genome of *Castanea dentata* is deficient in naturally occurring alleles for strong resistance. Thus, the specific objective of the breeding program of TACF is to produce backcross trees that will fall within the range of morphological, developmental, and ecological characteristics of *Castanea dentata* as understood from monographs and voucher specimens. It is anticipated that the B₃F₃ generation may meet this objective
- The goal of TACF will not be achieved by replacing the existing millions of surviving American chestnuts with B₃F₃ trees. Plantings may never be established in some rather large blocks of the chestnut range, such as the Shennandoah and Smoky Mountain National Parks. Also, natural regeneration from planted trees, with accompanying natural selection and the potential for hybridization with native chestnut, will play large roles in achieving the goal of TACF. Finally, it is expected that additional sources of blight resistance must be incorporated into the breeding program, and it is expected that additional backcross generations may be warranted to achieve a higher average proportion of American chestnut alleles in the genome.
- For these reasons, future breeding will be required to bring new sources of resistance into the breeding pool and carry the existing backcrosses to additional generations. Also, it is expected that future breeding programs will place ever increasing emphasis on regional adaptation by employing local, autochthonous sources of American chestnut parents.
- The purpose of testing the B₃F₃ generation is to determine how well we have progressed toward our goal at a stage that is expected to yield a tree that bears strong resemblance to *Castanea dentata* and has good resistance to blight. In other

words, the principal objectives of testing are to determine 1) to what degree the B_3F_3 resembles American chestnut, especially in a natural forest setting, and to what degree Asiatic characteristics (other than blight resistance) may remain, 2) to what degree the B_3F_3 is resistant to blight, and 3) how long resistance persists in B_3F_3 plantations. Subsidiary objectives are to determine if there are differences in performance among the progeny sets of different B_3F_2 parents, to measure the extent of genotype x environment interaction, and to identify differences in regional adaptability as suggested by growth, survival, and phenology.

- Although test plantations might later be converted to seed orchards, test plantations should not be designed with the purpose in mind of converting them to seed orchards at a later date. The creation of seed orchards should be pursued separately from testing.
- Testing should precede public distribution or sale of seed with implied genetic qualities.
- Future breeding and deployment efforts should be guided by test results.

Task Force Recommendations:

- Testing will involve finding and preparing planting sites, getting labeled trees from TACF, planting them, mapping their locations, protecting them, and measuring them. It is expected that test plantations will be arranged, installed, maintained, and measured by chapter volunteers and other cooperators.
- Given the goal of the breeding program, testing shall be done on:
 - naturally forested sites (which may be temporarily devoid of trees because of recent harvest),
 - on soils that are considered suitable for American chestnut, and
 - in close (a few hundred yards maximum) proximity to existing sprouts of American chestnut.

This latter requirement, which may not always be possible to meet, ensures that the soils are appropriate for American chestnut, provides the opportunity of directly comparing phenology of backcross trees as one indicator of environmental adaptation, and provides the opportunity to determine whether resistance alleles from TACF backcrosses can move into other American chestnut by natural crossing.

- To the extent possible, test sites shall be scattered throughout the original distribution of *Castanea dentata*, or at least the region of its greatest abundance

(Massachusetts to North Carolina and Tennessee). Although B_3F_3 material now under development was derived from central Appalachian provenances of American chestnut, it is important to learn about regional adaptability in this species and in the likely products of the TACF breeding program.

- Test sites should be evaluated and approved prior to planting by someone from TACF. Corner coordinates shall be established by accurate GPS measurement.
- Cooperators shall agree to the use of standard protocols for the design and measurement of test plantations. It may be desirable and even necessary to underwrite these tests with research grants from TACF.
- Test plantation design shall conform to the following criteria:
 - 8- x 8-foot spacing between trees (square grid arrangement),
 - minimum of 25 single-tree replications of each experimental unit (“treatment”) (see below), and
 - completely randomized design.
- Each test plantation shall contain the following experimental units:
 - pure American chestnut of local provenance at double replication (minimum 50),
 - one or more designated Asian chestnut varieties at double replication (minimum 50),
 - a core set of at least five B_3F_3 families (open-pollinated progenies of B_3F_2 lines) common to all plantations, and
 - additional B_3F_3 families as availability and space allow, including if possible families from different regional breeding programs.
 - advanced generation backcrosses (other than B_3) if available.
- Plantation sites shall have minimal overstory (30 sq. ft./acre basal area), and clearcut sites will be generally preferred. Existing green vegetation shall be sprayed with glyphosate herbicide in a 1-meter circle around each planting spot in advance of planting. Directed applications of glyphosate (with seedlings shielded) shall be used after plantation establishment as needed to exclude serious competition from herbaceous and woody plants. The site shall be protected from deer if deer browsing is likely to be serious.
- All records of test plantation establishment, including a map of the design, detailed directions to the location, contact information for ownership, date of establishment, and contact information for the person responsible, shall be sent to a central office to be designated by TACF.

- Cooperators shall commit to making annual (initially) measurements of the following:
 - height,
 - diameter,
 - survival,
 - form (index to be developed),
 - severity of cankering (0 = none),
 - date of bud burst,
 - date of flowering,
 - date of fruit maturation,
 - date of fall coloration,
 - abundance of male and female flowers (0 = none), and
 - presence and nature of other serious insect or disease injury.

For reference, the dates of phenological events (bud burst, flowering, fruit maturation, and fall coloration) should also be recorded for nearby native trees. It is highly recommended that plantations be visited several times during the growing season, especially during the first few years.

Measurement protocols will be developed and distributed to cooperators. Cooperators may record additional information if they desire.

- In order to allow measurements of naturally occurring rates of disease progress, trees will not be artificially inoculated. Other plantings in orchard settings will be tested for blight resistance by artificial inoculation.
- Cooperators shall verbally commit to plantation care and measurement for a minimum five-year period. All data shall be sent annually to the central office designated by TACF. Cooperators shall be free to publish data from their test plantations. The central office will annually prepare a report on the progress and performance of all test plantations. It is impossible to specify a precise duration for the usefulness of these test plantations. A minimum of three to five years' evaluation will be required for preliminary conclusions about relative performance. Fairly definitive conclusions about some aspects of performance should be available within ten years. With time, the value of each additional year's duration will diminish, but never quite to zero. A key question to be answered is whether backcross hybrid chestnuts will have the ability to grow to dominant canopy height in competition with naturally occurring trees.

- Test plantations will be thinned at an appropriate time after crown closure in order to maintain reasonable access with measuring poles without modifying too much the natural progression of forest stand development. When fairly definitive conclusions are possible regarding the relative performance of backcrosses vs. Asian chestnuts, a decision will have to be reached on whether to remove all Asian chestnuts from the test plantation. The purpose of this removal would be to remove a source of major pollen contamination from the planting.