

The American Chestnut Foundation Breeding Plan: Beginning and Intermediate Steps

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Introduction

The American Chestnut Foundation intends to restore the American chestnut, *Castanea dentata* (Marsh.) Borkh., as a viable component of our eastern hardwood forests. The primary breeding approach we use is to backcross the blight resistance of Chinese chestnut, *Castanea mollissima* Blume, into the American species³. The basic plan is to breed hybrids of the two species, cross these back three times to the American parent, and intercross the B3s to recover trees homozygous and true-breeding for blight resistance. We will select seedlings for blight resistance from among the backcross and intercross progeny. We will not attempt any further improvements to the American chestnut until we are certain the backcross method will succeed, and even then, we would limit improvements to those characteristics which are absolutely necessary to restore the species in order to retain as much genetic diversity as possible. Improving more conventional aspects of the American chestnut, such as increased growth rate or improved wood characteristics, could be undertaken with trees which we have released to the public.

We are using conventional breeding methods and this paper details how we screen progeny for blight resistance, where and how we breed trees, and how many progeny we produce within each backcross and intercross generation. This paper also explains how many lines of American chestnut we plan to carry, and the number of sources of Chinese chestnut we will use. The final steps of the program include testing the performance of B3-F2 or B3-F3 progeny in forest situations and developing methods for introducing suitable trees back into the forest, but these steps we will discuss at a later time.

Screening Progeny for Blight Resistance

Because current evidence indicates that trees with intermediate to low levels of blight resistance, such as backcross progeny, cannot be distinguished reliably by direct inoculation until they are 2.5 cm in DBH⁵, we begin screening for blight resistance when they have achieved this diameter at about 4 to 5 years of age.

In contrast, trees with high to intermediate levels of blight resistance, such as what we expect from intercross progeny, cannot be distinguished by direct inoculation if they exceed 1 cm in DBH (Hebard & Shain, unpublished); thus, intercross progeny are screened when they are 2 years old using the micro-direct inoculation technique appropriate for smaller trees.

The direct inoculation technique entails removing a plug of bark from a tree and inserting a disk of agar of mycelium of the chestnut blight fungus into the hole. In the micro-direct method the hole is smaller, about 1 to 2 millimeters in diameter versus 4 - 6 millimeters in diameter for the regular method.)

Trees with intermediate levels of blight resistance are being screened for resistance at 4 to 5 years of age; they are planted at 20-foot by 7-foot spacing. Trees with high levels of resistance are screened at 2 years of age, planted at 10-foot by 2-foot spacing. We are using these orchard spacings to eliminate crowding of trees at the time they are screened for blight resistance. These spacings were determined based on experiments done by Uchida¹⁴ with Japanese chestnut, *Castanea crenata* Sieb. & Zucc. He found that crowded trees were more susceptible to blight than uncrowded trees; thus, crowding might hamper our ability to distinguish resistance classes.

Because progenies are not screened for blight resistance until they are several years old, seeds are planted directly.⁷ Compared to transplanting seedlings from the greenhouse or nursery, direct seeding at

orchard spacing results in faster plant growth and requires much less labor.

In the last three years more than 80 percent of nuts have sprouted and developed into viable seedlings. Many seedlings, including pure American chestnut, are bearing male flowers at 1 to 3 years of age, and female flowers at 3 to 4 years.

Trees planted within orchards are arranged in a statistically random fashion. American and Chinese chestnut, their first hybrid and Chinese chestnut cultivars- 'Nanking', 'Meiling', or 'Kuling'-are also planted in the orchards to serve as standards for evaluating the blight resistance of progeny from crosses. Six to ten control plants of each type are planted for every 500 trees.

After trees have been screened for blight resistance, most will be cut down to reduce the amount of blight fungus at the farm. Undesirable trees will be removed as appropriate for space and experimental needs while selected trees will be allowed to resprout. However, those select trees which appear ready to flower will not be pruned, although cankers with blight stromata will be excised.

Pollen is collected from selected trees as soon as they flower. To speed up production of the next generation, selected trees will be used primarily as pollen parents until the intercross generation. This speeds up production because numerous female flowers can be pollinated by one catkin, whereas female flowers yield only one to three nuts because seedling chestnut trees generally bear male flowers prior to female flowers, and because chestnut trees generally bear many more male flowers than female flowers.

How and Where Trees Are Bred

To maintain adaptations to local conditions and enable us to start breeding trees immediately, as many crosses as possible have been being made on flowering American chestnut sprouts growing at their original locations. Hand pollinations are employed using methods described by Rutter¹¹. Currently in Pennsylvania, Virginia, North Carolina, South Carolina, Kentucky, Tennessee and Georgia, numerous flowering trees occur in clearcuts, and other disturbed areas, in the national forests. The flowering period of most chestnut trees occurs between 5 and 10 years after the overstory of other tree species has been removed.

Flowering is terminated by blight acting together with suppression of new sprouts by competing vegetation. Thus, removing competing vegetation in cutover areas could prolong the flowering period and greatly increase the yield of nuts. In addition to pollinating these trees, we also are planting American chestnut seed, and transplanting naturally occurring seedlings and sprouts at easily accessible locations. Additionally, the ACF distributes American chestnut seed to members so they can raise mother trees for breeding.

Controlled hand-pollination of chestnut is very labor intensive, generally about one nut is produced per pollination bag, and one person does well to place 200 bags in a 12-hour period. There are several alternative methods of producing controlled-pollination progeny which would be much less labor intensive.

First, desirable plants could be grafted onto rootstock in clearcut areas where there is an abundance of American chestnut sprouts (it is necessary to graft at ground level and cover the graft union with soil to exclude blight). Seed from these plants would be pollinated by nearby American chestnut trees.

Second, outside the natural range of chestnut, isolated pairs of trees could be planted; the two trees in the pair making a desirable cross. The composition of the cross could be altered by grafting scions of another tree into one member of the pair.

Third, in the Midwest there are large, isolated American chestnut trees already growing; scions from other trees could be grafted into the crowns, and ungrafted portions of the crown would supply pollen to the grafts and vice versa.

Fourth, in the East some American chestnut trees survive blight due to hypovirulence, and many of these flower. If the American chestnut tree is reproductively isolated, nearby Chinese chestnut trees could be grafted with American chestnut above ground level. In the East one can graft above ground level into Chinese chestnut trees, but not into American chestnut trees because blight will rapidly invade the graft union.

One disadvantage of these hands-free methods of pollination is that they take several years to begin producing progeny, but then produce abundantly. Since we do not want a single American chestnut tree to dominate our backcross pedigrees, many of the methods probably would be of limited use in the backcrossing stage of our program, although they could very useful at the intercrossing stage. Additionally, every year we need F1 seed to plant as controls, and it can be difficult to avoid pollen contamination with

bagged progeny. The hands-free methods could fill this immediate need very well.

How Many Progeny from Each Cross

The number of progeny needed for each cross is calculated based on the number of genes believed to confer blight resistance. Current evidence indicates two genes control blight resistance in Chinese chestnut, and several elements point to this conclusion.

Evidence from Clapper's⁴ report shows that about 14 of progeny from a backcross of an F1 Chinese American hybrid back to Chinese had high levels of blight resistance.

Also, the 'Clapper' first backcross⁸ and undescribed 'Graves' first backcross¹ show levels of resistance comparable to F1 hybrids-these hybrids were selected from no more than 50 siblings each.

Finally, large populations of F2 and B1-F2 progeny at the ACF's Meadowview farm are being screened for blight resistance (the trees were inoculated in June 1993), and results show that blight resistance is controlled by one to three major genes, probably two. We expect additional evidence will come from the results of screening our backcross progeny for blight resistance, which is due to begin this year.

Until the number of genes controlling blight resistance is verified, however, our plans assume that resistance is controlled by three genes, an approach that provides a safe margin of error for the program.

In each backcross generation we want to obtain four or five nuts which carry all major genes for blight resistance. From these nuts we should be able to grow two to three trees and select the most American-type trees.

Growing 73 backcross progeny will give us a 99 percent chance of obtaining at least four plants with the three genes, according to the following binomial formula:

$$p = 0.99 = 1 - \sum_{m=0}^3 \binom{74}{m} * 0.125^m * 0.875^{(74-m)}$$

For intercross (F2) progeny, the same formula indicates (after substituting in the appropriate numbers) that 149 intercross progeny must be grown to be 99 percent certain of obtaining four plants that will be homozygous for two resistance genes.

However, it would be desirable to produce even more progeny of each cross because then we could select among blight-resistant progeny for American traits, thus by-passing several generations of crosses needed to recover American chestnut traits.² But the space and time requirements of the current method of screening trees for blight resistance make it impractical to grow many more progeny than we presently do.

A genetic molecular marker approach is attractive in that we might be able to screen offspring using samples of nut meat or true leaves from freshly germinated nuts. Currently we can generate numerous offspring once trees start flowering, but the rate at which we can advance the breeding program is limited by the time it takes trees to flower.

On the other hand, the molecular marker approach may be too expensive for routine use. Also, molecular markers provide indirect evidence of blight resistance, rather than the direct evidence provided by inoculation. If we use them, we will have to be very careful to ensure that major genes for blight resistance are mapped accurately and that linkages to minor genes are not missed altogether.

However, if there are more than three genes for blight resistance, molecular markers linked to each gene could be used to help backcross them separately into American chestnut, in parallel. The genes from the parallel backcrosses would be combined after backcrossing was complete in order to recover highly blight-resistant trees. It would be preferable if each of these blight resistance genes were detectable by direct-inoculation tests to ensure precise mapping.

Marker-directed selection would also be useful in combining, (known as pyramiding), the genes conferring low levels of blight resistance in large, surviving American chestnut trees with the hope of obtaining trees with high levels of blight resistance. Using markers to follow the process, relatively few plants might be needed compared to the number of plants needed under conventional breeding methods, such as recurrent selection for phenotype. Thor has outlined a conventional breeding program for large, surviving American chestnut trees.¹²

Micropropagation is another tool of biotechnology which could be useful to our program.

Micropropagation, cloning chestnuts at the bud stage in tissue culture using plant growth hormones, of highly blight-resistant B3-F2 (or earlier F2 stages) trees would facilitate evaluating their performance in the field. The technology for micropropagation is immediately available for the small-scale use envisioned here. Micropropagation of selected B3-F2 nuts would also accelerate and increase the production of highly blight-resistant B3-F3 nuts which we intend to use as our primary vehicle for distributing blight-resistant progeny.

How Many Lines of American Chestnut

The key question is how many lines of trees to advance. Namkoong¹⁰ estimated that "A few thousand samples are needed to save most alleles in most populations..." In alfalfa, which is a cross-pollinated plant like chestnut, 125 lines were used by Stanford and Houston¹³ in backcrossing resistance to bacterial wilt, mildew and leaf spot into 'California Common' to produce the Caliverde variety. We can handle 60 breeding lines at the Meadowview facility. Five additional breeding locales advancing 20 lines each would give us 160 lines.

The contrast between 160 lines and Namkoong's estimate of a few thousand samples makes it clear that the genetic diversity of our products will be less than that which existed prior to blight. It is also clear that there cannot be too many locales where American chestnut trees are bred for blight resistance!

Our current breeding program is concentrated in the vicinity of Meadowview, Virginia, but our goal is to restore the American chestnut throughout its native range. Thus, to preserve adaptations to local conditions, we hope to replicate at least part of the Meadowview breeding effort every few hundred miles from Maine to Georgia. Alternatively, we could breed trees adapted to local conditions by backcrossing highly blight-resistant B3-F2 trees from Meadowview into local populations followed by a large intercross generation. However, this might require long-term testing to select trees adapted to the local conditions. A few additional backcrosses to locally adapted American chestnut trees prior to intercrossing is a more rapid, but more labor-intensive means of achieving this goal.

A breeding line of American chestnut is defined here as the product of one intercross of a Chinese chestnut tree and an American chestnut tree and three backcrosses to American chestnut. For each backcross within a line, the American chestnut parents would be separate individuals, in order to avoid inbreeding. Thus, one Chinese and four American chestnut trees would be the parents of one line. After three backcrosses, the progeny will have to be intercrossed. We will probably intercross between lines, but within sources of Chinese chestnut resistance.

The 1 to 4 ratio of Chinese to American parentage is the basic concept behind our breeding lines, however, in the field the makeup is somewhat complicated. In actuality, more than four American parents may be involved in the makeup of each line because we need 73 progeny per line at each backcross step, but we cannot generally obtain this many offspring from any single flowering American chestnut tree in a typical clearcut. Thus, we have decided to equate each managed area, referred to as a clearcut, to a single American parent. We will try to have the clearcuts which are the "parents" of a single line be no more than 10 km apart, and at similar elevations. Due to blight, generally it is not possible to use American chestnut trees in a clearcut for more than one backcross generation.

Each clearcut may include multiple Chinese parents, and thus multiple sources of resistance. We are keeping pedigrees and noting when progeny from different Chinese parents have the same American parent tree from a clearcut; use of the same American tree with different Chinese sources occurs infrequently because most American chestnut trees in clearcuts bear only one crop of nuts before succumbing to blight.

How Many Sources of Chinese Chestnut Resistance

The purpose of backcrossing is to recover all characteristics of the recurrent parent except for the trait being transferred from the donor parent. Thus a high level of blight resistance is the only characteristic we use in evaluating Chinese, and other chestnut trees, as sources of blight resistance. For backcrossing, the best sources of resistance are those which confer the most resistance with the fewest genes. Sources are evaluated using the direct inoculation technique to compare their resistance, and that of F1, F2 and backcross progeny. Where possible, F2 and backcross progeny from various sources will be interplanted so their performance can be compared.

If two Chinese chestnut cultivars have identical genes for resistance, creating separate sets of lines for each cultivar would be redundant. We will try to determine whether Chinese chestnut cultivars have

identical genes for resistance, hopefully by examining progeny with molecular markers. Molecular markers should at least tell us whether major blight resistance genes are mapped closely together if they cannot tell us whether they are multiple alleles for resistance at the same location.

We have room to carry three sources of resistance in 20 lines each at the Meadowview facility. Twenty lines are chosen as the absolute minimum number necessary to preserve genetic diversity on the American side for a single source of resistance.

Three sources of resistance currently have the highest priority at the Meadowview facility. The first source is the triplet of Chinese chestnut cultivars, 'Meiling', 'Nanking' and 'Kuling.' These three cultivars are considered a single source of resistance because they came from the same, or very similar seed lots.⁹ They have high priority because in contrast to many Chinese chestnut trees⁶ they have demonstrated high levels of blight resistance: there are few, if any, blight cankers on most trees of these cultivars. We have advanced one line of 'Nanking' to B1.

The other two sources of resistance are the 'Graves' and 'Clapper' first backcrosses. The 'Graves' and 'Clapper' sources have high priority because they are our most advanced breeding lines and we wish to prove the utility of the backcross method. We are beginning to advance these to B3 in blind crosses. Unfortunately, we have only one line of American chestnut in the 'Graves' and 'Clapper' trees. It will be necessary to broaden their genetic base into 20 American lines. This probably will require one or two additional backcross generations. The Chinese grandparent of the 'Graves' tree is still living, as well as some F1 hybrids between it and American chestnut. These trees can provide additional lines for the 'Graves' source.

Ideally, we would like to have perhaps 100 individual Chinese chestnut trees comprising our source(s) of blight resistance, in order to maintain genetic diversity at the Chinese genes which remain in our final breeding products. A more realistic figure will probably be ten or twenty because of the pressing need to use numerous American parents.

Conclusion

Editors Note: This paper explains in detail how we are breeding Chinese and American chestnut trees to produce a blight resistant chestnut that also looks like our American native. As you can see from this article it is a process requiring careful attention to detail from a whole host of perspectives. One way you can get a better sense of the progress we have made at Meadowview is to come and visit! The Wagner Research Farm is open to the public year-round and those who plan longer visits can get a hands-on sense of chestnut breeding by helping to pollinate trees in the summer months. For more information on visits and volunteer efforts, please contact Fred Hebard at the farm.

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