The American Chestnut Foundation Breeding Plan: Beginning and Intermediate Steps

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ABSTRACT. American chestnut trees are being bred for blight resistance using traditional breeding methods. Trees with intermediate levels of blight resistance are being screened for resistance at 5 yr of age; they are planted at 6 x 2 m spacing. Trees with high levels of resistance are screened at 2 yr of age, planted at 3 x 0.5 m spacing. Trees are screened for blight resistance by the direct inoculation method. Nuts are planted directly at orchard spacing to save time, money and to accelerate plant growth. Germination rates were about 85% in 1991. In 1991, male flowers developed on more than 50% of American chestnut and American-Chinese hybrids planted in 1989. Female flowers are expected to develop 5-8 yr after planting. The cost of equipment, supplies, labor and land to produce, plant, tend and screen 2000 seedlings per yr is $20,000 plus the salary of one scientist. Using the backcross method to transfer the blight resistance of Chinese chestnut to American chestnut, 15/16th American chestnut trees that are homozygous for resistance are expected to begin fruiting 15 yr hence. However, it is still not certain how many major genes confer blight resistance in Chinese chestnut. If there are less than four, no additional technical knowledge will be needed to complete the breeding. Additional numbers of genes might make the backcross method impractical using only classical methods of plant breeding. Molecular biologists could assist the breeding work by developing RFLP or RAPD markers that span the chestnut genome. This would allow selection for numerous American chestnut characteristics at each backcross step, accelerating the elimination of undesirable traits from the Chinese parent. Markers closely linked to blight resistance loci might eliminate a need for blind crossing in the backcross program. Markers for blight resistance also would allow selection of backcross nuts that were homozygous for resistance at the F2 stage; this would allow distribution of progeny at the F2 instead of the F3 stage, skipping one 6-10 yr generation. The large surviving American chestnut trees would be very useful sources of genes for breeding later generations of trees. Progeny also could be distributed at the F2 stage using micropropagation methods. Micropropagation would be especially useful at early stages of the breeding program in multiplying highly blight-resistant, F2 progeny for testing in the forest. The breeding work will assist molecular biologists by providing materials from which the genes for blight resistance might be cloned.

PLAN

The American Chestnut Foundation (ACF) intends to restore the American chestnut, *Castanea dentata* (Marsh.) Borkh., as a viable eastern hardwood tree. The primary approach being pursued is to backcross the blight resistance of Chinese chestnut, C. *mollissima* BI., into the American species. The rationale for this approach has been published previously (1). The basic plan is to hybridize the two species, cross back three times to the American parent, and intercross the B_as to recover trees homozygous and true-breeding for blight resistance. We will select for blight resistance among the backcross and intercross progeny. We do not plan to attempt additional improvements to the tree until we are certain the backcross method will be successful. Even then, we would limit improvements to those absolutely necessary to restoring the species, so that genetic diversity can be retained. Improvement of American chestnut in more conventional aspects, such as increased growth rate or improved wood characteristics could be undertaken with trees that we have released to the public.

This paper details how to screen progeny for resistance, where and how to breed trees, how many progeny to produce within each backcross and intercross generation, how many lines of American chestnut to carry, and how many sources of Chinese chestnut to use. The final steps in the program are to test the performance of B3-F2 or B3-F3 progeny in forest situations, and to develop methods for introducing suitable trees back into the forest. These steps are not discussed.

Screening progeny for blight resistance. Backcross progeny will be screened for blight resistance using the direct inoculation technique, when they are 4-5 yr old and have a minimum diameter of 2.5 cm dbh. Current evidence indicates that trees with low to intermediate levels of blight resistance, such as backcross progeny, cannot be distinguished reliably by direct inoculation until they are 2.5 cm dbh (3). In contrast, trees with intermediate to high 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 and Shain, unpublished). Thus, intercross progeny will be screened when they are 2 yr old. The micro-direct inoculation technique will be used for the smaller trees (3).

Backcross progeny are being grown at 6 x 2 m spacing since they will be screened for blight resistance at 5 yr of age, whereas intercross progeny, screened for resistance at 2 yr of age, are being grown at 3 x 0.5 m spacing. These spacings were chosen to eliminate crowding of trees at the time of screening. The spacings are based on the experiments of Uchida (12) with Japanese chestnut *(C. crenata* Sieb. and Zucc). He found that crowded trees were more susceptible to blight than uncrowded trees; thus, crowding might hamper our ability to distinguish resistance classes.

Since progenies will not be screened for blight resistance until they are several years old, seed are being planted directly at orchard spacing, using previously described methods (5). Compared to transplanting seedlings from the greenhouse or nursery, direct seeding results in faster plant growth and requires much less labor. Emergence and survival over the first growing season have exceeded 80 percent for the past 2 yr. Many seedlings are bearing male flowers at 1-3 yr of age.

Orchards are being planted in completely randomized designs. American and Chinese chestnut, their first hybrid and the Chinese chestnut cultivar,`Nanking,' are being 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 being planted per 500 trees.

After trees have been screened for blight resistance, they will be coppiced (pruned at ground level) to reduce inoculum in the orchard. Undesirable trees will be rogued, to create space for future experiments. Selected trees that appear ready to flower will not be coppiced, although cankers with stromata will be excised. Pollen will be 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 will speed 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 before female flowers, and because chestnut trees generally bear many more male flowers than female flowers.

How and where to breed? To maintain adaptations to local conditions and enable us to start breeding now, as many crosses as possible are being made on flowering American chestnut sprouts growing at their original location. Hand pollination are employed using methods described by Rutter (9). Currently, in Pennsylvania, Virginia, North Carolina, South Carolina, Kentucky, Tennessee and Georgia, numerous flowering trees occur in clearcuts in the National Forests. The flowering period of most trees occurs between 5 and 10 yr after clearcutting. It is terminated by blight and suppression of new sprouts by competing vegetation. Removal of competing vegetation could prolong the flowering period. We also are planting American chestnut seed at central locations and transplanting naturally occurring seedlings and sprouts to central locations. Additionally, the ACF is distributing American chestnut seed to members for the same purpose.

There are some hands-free methods of producing controlled-pollination progeny that could be employed. First, desirable plants could be grafted onto rootstock in clearcut areas with abundant American chestnut regeneration (it is necessary to graft at ground level and cover the graft union with soil to exclude blight). Seed from those plants would have been pollinated by the 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. Such trees could be top-worked (grafted) to alter the cross. Third, scions from desirable trees could be grafted into the crown of large isolated American chestnut already growing in the Midwest; 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 flower. If they are reproductively isolated, nearby Chinese chestnut trees could be top-worked with desirable trees (one cannot top-work American chestnut trees in the east due to blight in the graft union).

How many progeny from each cross? Before estimating the number of progeny needed for each cross, it will be necessary to know the number of genes that confer blight resistance. Current evidence indicates that blight resistance in Chinese chestnut is controlled by two genes. This evidence is the report of Clapper (2), and the existence of the 'Clapper' first backcross (6) and the undescribed `Graves' first backcross, which have levels of blight resistance comparable to that of F1 hybrids and which were selected from no more than 50 siblings each. Large populations of F2 and B_1-F2 progeny are currently being grown at the ACFs Meadowview farm to provide additional evidence for the number of genes controlling blight resistance. We also will obtain evidence from the results of screening backcross progeny for blight resistance.

Until the number of genes controlling blight resistance are verified, we will plan for three genes, since many years could be lost without such planning. It would be better to have four or five plants in each backcross generation that carry all the resistance genes. In backcrosses, growing 73 progeny will give us a 99 percent chance of obtaining at

least four plants with the three genes, according to the following binomial formula:

$$p = 0.99 = 1 - \Sigma_{m=0}^{3} \binom{74}{m} \times 0.125^{m} \times 0.875^{74-m}$$

For intercross (F2) progeny, the same formula indicates (after substituting the appropriate numbers) that 149 intercross progeny have to be grown to expect four plants homozygous for two resistance genes, with 99 percent certainty.

If we can develop numerous markers to distinguish between Chinese and American chestnut, such as restriction fragment length polymorphisms (RFLP), it would be desirable to produce more progeny of each backcross. Then we could select among blight-resistant progeny for American characteristics. This would reduce the number of backcross generations needed to recover American-type chestnut trees. A limiting factor in the speed of breeding, at present, is the time it takes American chestnut trees to produce male flowers. Once they do flower, we quickly can produce large numbers of progeny. These considerations make the RFLP approach very attractive, if one can grow and probe the many samples needed. Automated equipment designed to probe large sample sizes is being developed by the Human Genome Project (David Burke, personal communication).

If we can find markers closely linked to blight resistance loci, then we might be able to screen freshly harvested nuts for blight resistance by testing samples of cotyledons. This would enable us to distribute blight-resistant nuts at the B3-F2 stage instead of the B3-F3 stage. At present, we probably will not want to rely on marker-directed selection for blight resistance at earlier steps in the breeding process, except when trees flower prior to being screened for resistance by direct inoculation. However, if there are more than two genes for blight resistance, markers linked to each of them could be used to help backcross each gene separately into American chestnut, in parallel. It would be preferable if each blight resistance gene were detectable by direct-inoculation resistance test. The genes would be recombined after American-type chestnut trees were recovered.

Marker-directed selection also would be useful in pyramiding the genes conferring low levels of blight resistance in large, surviving American chestnuts. Relatively few plants might need to be grown to achieve fairly high degrees of pyramiding. Thor has outlined a conventional breeding program for large, surviving American chestnut trees (10).

Micropropagation would be useful for cloning B_3-F_2 (or earlier F2 stages) trees that were highly blight resistant, in order to evaluate their performance in the field. The technology for micropropagation is immediately available for the small-scale use envisioned here.

Micropropagation also might be useful for accelerating the production of B3-F3 nuts, since more parents would be producing. We intend to rely on nuts produced by selected B_3-F2 trees as our primary vehicle for distributing blight-resistant trees.

How many lines of American? 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 cross within a line, the American chestnut parents would be separate individuals. Thus, one Chinese and four American chestnut trees would be the parents of one line. In most cases, each line would have separate American parents from other lines. After three backcrosses, the progeny will have to be intercrossed. We have not decided yet whether to intercross progeny within lines or between lines.

In actuality, more than four American parents may be involved in the makeup of each line. This is because we need 73 progeny per line at each backcross step, but we cannot generally obtain 73 progeny from a single flowering American chestnut in a clearcut. Thus, we are equating each clearcut to a single American parent. We will try to have the clearcuts that are the "parents" of a single line be no more than 10 km apart, and at similar elevations. Due to mortality, it generally is not possible to use American chestnut trees in a clearcut for more than one backcross generation. The trees in each clearcut are being used as parents for more than one line of American chestnut trees, but the lines have different Chinese chestnut parents. We are keeping pedigrees for our progeny and noting when progeny from different Chinese parents have the same American parent from a clearcut; this occurs infrequently because most American chestnut trees in clearcuts bear only one crop of nuts before succumbing to blight.

Our current breeding is concentrated in the vicinity of Meadowview, Va., but our goal is to restore the species throughout its 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 locally adapted, blight-resistant trees.

The key question is how many lines of trees to advance. Namkoong (8) 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 Houston (11) 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. So it is clear that the genetic diversity of our products will be less than that which existed prior to blight. It also is clear that there cannot be too many locales where American chestnut trees are bred for blight resistance!

How many sources of Chinese chestnut resistance? The purpose of backcrossing is to recover all characteristics of the recurrent parent except for the character(s) being transferred from the donor parent. Thus, a high level of blight resistance is the only characteristic we will use in evaluating Chinese chestnut trees as sources of blight resistance. The best sources of resistance are those that confer the most resistance with the fewest genes. Finding the best sources is a high priority at present. Sources will be 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. We plan to carry three sources of resistance in 20 lines each of American chestnut at the Meadowview facility.

We will attempt to determine whether Chinese chestnut cultivars have identical genes for resistance, hopefully using RFLP or RAPD methods (RAPD is an acronym for random amplification of polymorphic deoxyribonucleic acid). These methods should at least tell us whether major blight resistance genes are near the same locus, if they cannot inform us of the existence of multiple resistance alleles.

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 as a single source of resistance since they came from the same, or very similar seedlots (7). They have high priority because, in contrast to many Chinese chestnut trees (4), 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 B_1.

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 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 loci that remain in our final breeding products. A more realistic figure will probably be ten or twenty.

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