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THE BACKCROSS BREEDING PROGRAM OF THE AMERICAN CHESTNUT FOUNDATION

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Abstract: The blight resistance of oriental chestnut trees is being backcrossed into American chestnut using traditional plant breeding techniques. Progeny are screened for blight resistance by direct inoculation with the blight fungus, when they are old enough to survive inoculation, which is 3 or 4 years for trees with intermediate levels of blight resistance, and 1 or 2 years for trees with high levels of blight resistance. Trees are grown using intensive horticultural techniques. Probably the most unusual aspect of this breeding program in comparison to similar programs for crop plants is the large acreages over which trees are grown, and the fact that the objective is recovery of a genetically diverse species rather than an improved cultivar. Highly blight resistant progeny have been recovered from intercrosses of straight F_1s, B_1s and B_2s, suggesting strongly that it should be possible to backcross blight resistance into American chestnut. Currently, two sources of blight resistance are being advanced to B_3-F_2. These are expected to begin producing progeny suitable for outplanting within 2 to 3 years.

INTRODUCTION

The American chestnut tree, *Castanea dentata* (Marsh.) Borkh., has been destroyed as a dominant forest tree by a canker disease, chestnut blight, incited by *Cryphonectria parasitica* (Murr.) Barr. The blight fungus was introduced into eastern North America around the turn of the 20th Century, probably in blight cankers on imported Japanese chestnut, *C. crenata* Sieb & Zucc., nursery stock (Metcalfe and Collins, 1909). By 1950, the disease had killed almost all of the large American chestnut trees throughout their range.

By 1930, when the American chestnut was thought to be doomed, attempts had begun to breed blight-resistant replacements. These attempts were abandoned, for the most part, around 1960, when no trees had been developed that combined the blight resistance of oriental chestnut trees with the large size of American chestnut trees (Jaynes, 1994).

In 1961, what later proved to be viruses (Hillman *et al.,* 2000) were found infecting *C. parastica* (Grente, 1961). The infected strains had been isolated from blight cankers on European chestnut trees, *Castanea sativa* Mill., growing in Italy. The viruses reduced the virulence of the blight fungus enough that infected strains could no longer kill European chestnut trees. Additionally the viruses spread from one canker to another, resulting, apparently, in the protection of entire stands of European chestnut. When viruses were introduced into blight cankers on European chestnut in France, the disease there was ameliorated. This discovery led to efforts to control blight on American chestnut with these viruses, which continue today. To date, the results of this effort have not been entirely satisfactory (Anagnostakis, 1990).

In 1981, Charles Burnham proposed that the blight resistance of oriental chestnut trees, primarily Chinese chestnut, *Castanea mollissima* Blume, could be backcrossed into American chestnut. For American chestnut, this was a new method of plant breeding that had not been used in previous attempts to develop blight-resistant, timber-type chestnut trees. In 1983, The American Chestnut Foundation was established as a not-for-profit corporation to help fund work on Burnham's proposal (Burnham *et al.*, 1986). In 1989, the foundation had accumulated sufficient resources to hire a part-time researcher at a new research farm in Meadowview, VA, in the heart of the range of the American chestnut tree.

Subsequent to 1989, the foundation has grown to the point where it is supporting a large breeding effort in Meadowview, with four full-time workers tending trees on three farms totaling 130 acres. Additional workers are employed in Asheville, NC and at Penn State University to assist volunteer breeding efforts at eleven state chapters. The administrative headquarters in Bennington, VT, also supports volunteer breeding efforts in CT and VT. The purpose of this paper is to describe progress to date in this breeding program.

MATERIALS AND METHODS

Breeding Method

To transfer blight resistance from Chinese to American chestnut, individuals of the two species are first crossed. The progeny from this cross, first hybrids, or F_1s, usually are exactly one-half American and one-half Chinese chestnut. An F_1 is backcrossed to another American chestnut, decreasing the proportion of Chinese chestnut genes by a factor of one half, on average. The progeny of this second cross, the first backcross, are known as B_1s. Two more backcrosses again decrease the proportion of Chinese chestnut genes by a factor of one half each time, to one-eighth followed by one-sixteenth, on average, with the remaining fraction of genes being from the American parent.

At each step of backcrossing, resistant trees are selected by observing canker symptoms after inoculation of the progeny with the chestnut blight fungus (see below for details). The progeny also vary in the fraction of Chinese genes remaining, and selection against Chinese morphological type is made to accelerate recovery of the American type, using traits identified by Hebard (1995). Burnham estimated that three backcrosses to the American parent, with selection against Chinese morphological type, would be sufficient to recover trees that look and grow like the American chestnut of old.

The F_1 trees, and any subsequent backcross progeny, would be heterozygous, at best, for the genes conferring blight resistance. Thus they would not be true breeding for blight resistance, throwing both susceptible as well as resistant progeny. To recover trees homozygous for blight resistance, third backcross trees are intercrossed among themselves, so the progeny have a chance of inheriting the genes for blight resistance from both parents. The progeny of this first intercross of third backcross trees are known as B_3-F_2s.

Blight resistance is only partially dominant, so F_1s and backcrosses are, at best, intermediate in resistance between the two parent species. High levels of blight resistance, comparable to those found in the Chinese parent, are only recovered after intercrossing F_1 hybrids and backcrosses. This facilitates recovery of trees reasonably homozygous for blight resistance, since they test out as more resistant than heterozygotes.

To avoid inbreeding, and its consequent decrease in genetic diversity, a different American chestnut parent is used at each step of backcrossing. Thus, in an ideal situation, four American parents are used to produce a third backcross tree. The third backcross progeny from a unique set of four American parents are termed a recurrent parent line or line for short. At the intercrossing stage, more than one line is needed in order to minimize sib crosses and their resulting inbreeding. Hebard (1993) estimated that 20 lines would be needed to minimize loss of alleles from inbreeding. With four American parents per line, 20 lines require 80 separate American parents.

In practice, only one line was used until the first backcross with the ‘Graves’ and ‘Clapper’ sources of blight resistance. These two first backcross trees then were crossed with 20 American parents to yield the

second backcross generation, and with 20 additional parents to yield the third backcross. Thus the third backcross progeny are half first cousins rather than half third cousins.

To ensure that the progeny from intercrossing third backcross trees are homozygous for blight resistance loci, only one Chinese chestnut parent is used to make a set of 20 lines.

Sources of Blight Resistance

The availability of the named first backcross, 'Clapper' (Little and Diller, 1964), and the undescribed 'Graves' first backcross at the Connecticut Agricultural Research Station plantings in Hamden gave a jump start to the breeding program in 1989. These two first backcross trees were backcrossed again onto about 30 American chestnut trees each between 1989 and 1995 to yield second backcross trees, or B_2s. Thirty American chestnut lines of third backcrosses were produced between 1996 and 2003 for both the 'Clapper' and the 'Graves' lines. From 2001 until present, second generation third backcross progeny, or B_3-F_2s, have been collected and planted from intercrosses within sources of blight resistance. The Chinese chestnut grandparent of 'Graves' is an undescribed seedling known as 'Mahogany.'

In 1989, breeding also was started with the Chinese chestnut cultivar, Nanking, crossing it with 20 American chestnut trees to start 20 recurrent parent lines at F_1. Cultivar Nanking was chosen because it had shown the highest blight resistance of any Chinese chestnut tree evaluated by Headland and Griffin (1976) and was noted as having high blight resistance when first released.

As available, other Chinese and Japanese chestnut trees, and F_1 hybrids between these species and American chestnut, were crossed with American chestnut trees, in these later cases with only a few American chestnut trees rather than assembling 20 lines. Table 1 lists the sources of blight resistance at their most advanced stage of backcrossing as of April, 2004, and the number of American parent lines at the most advanced stage. As indicated above, additional lines occur at less advanced stages of backcrossing for some sources of blight resistance.

Table 1. Oriental sources of blight resistance being used at The American Chestnut Foundation's Research Farms in Meadowview, VA, their most advanced stage of backcrossing into American chestnut and the number of American parent lines at that stage as of April, 2004.

Source of Blight Resistance	**Stage of Backcrossing**	**Number of American Parent Lines**
Clapper	B_3-F_2	12
Mahogany	B_3-F_2	5
Douglas	B_3	2
Nanking	B_3	2
Sleeping Giant South Lot R11T14	B_3	1
Sleeping Giant South Lot R1T4	B_3	1
Sleeping Giant South Lot R1T7	B_3	3
Meiling	B_2	1
MusickChinese	B_2	2
Greg Miller 72-211	B_1	3
mollissima7	B_1	1

mollissima10	B_1	1
mollissima13	B_1	1
PI#104016	Japanese B_1	1
Dunstan seedling	F_1	1
FP7284	F_1	1
Greg Miller 65-18	F_1	3
Greg Miller 65-4	F_1	6
Kuling	F_1	4
Orrin	F_1	4
mollissima11	F_1	1
mollissima18	F_1	1
MAJ7Japanese	Japanese F_1	1
Jayne	mollissima x pumila	1
AbbsValley	Chinese	
Altamont	Chinese	
Armstrong	Chinese	
Eaton	Chinese	
MacBoyd	Chinese	
MAJ	Chinese	
MAJ4	Chinese	
MAJ5	Chinese	
Waynesboro	Chinese	
mollissima12	Chinese	
mollissima14	Chinese	
mollissima15	Chinese	
mollissima16	Chinese	
mollissima17	Chinese	
mollissima19	Chinese	
mollissima20	Chinese	
mollissima8	Chinese	
PI#7284	Chinese	
PI#97853	Chinese	
Richwood	Chinese	
Wilkinson	Chinese	
YardChinese	Chinese	
FPGlenDaleID:GS	Japanese	

American Chestnut Parents

In addition to the breeding at Meadowview, the American Chestnut Foundation also has an extensive network of state chapters staffed primarily by volunteers, and advised by staff officers stationed in North Carolina and Pennsylvania (Paul Sisco and Sara Fitzsimmons, respectively). The chapters have been crossing pollen of 'Graves' and 'Clapper' second backcrosses from Meadowview onto local American chestnut trees to produce third backcross trees, for the most part. The intent is to produce a viable breeding population of 20 individuals for each source of blight resistance, adapted to the local conditions, and also to increase the genetic diversity of the breeding population, as originally proposed by Inman (1987). Table 2 depicts the number of third backcross trees in the various states as of 2004.

Table 2. Number of third-backcross (B_3) chestnut at TACF breeding orchards in 2004, with the number of sources of blight resistance and the number of American chestnut lines in the breeding stock.

State	**Nuts or Trees**	**Number of Sources of Resistance**	**American Lines**
Maine	1445	2	29
Massachusetts	3076	2	28
Pennsylvania	5350	2	36
Maryland	33	1	1
Indiana	1496	1	11
Kentucky	150	2	2
Virginia (Meadowview)	5275	8	73
North & South Carolina	1049	2	9
Tennessee	745	5	6
Alabama	566	1	5
Total	**19179**		

Following Inman's recommendation (Inman, 1989), attempts have been made to limit the range of American chestnut parents to within 20 miles of each other in building local populations. This has been easier near Meadowview than elsewhere, since the required numbers of flowering chestnut trees can be found within such a small area.

Pollination

First hybrids and straight backcrosses are produced using the controlled pollination techniques described by Rutter (1991). Subsequent experience indicates that the best time to bag chestnut flowers for controlled pollination when the styles begin to emerge from the bur, rather than to assess the time by observing the onset of anthesis, as recommended by Rutter (1991). Experience also suggests that the slide technique using dried pollen described by Rutter (1991) to be more efficient than pollinating with fresh catkins. Flat surfaces other than microscope slides have been found preferable for applying pollen, such as the lid of the pollen container. In general, about one nut is produced per pollination bag placed over female flowers.

The intercross generations are produced by open pollination, where possible. Thus breeding orchards containing straight third backcross trees (B_3) from one sources of blight resistance are isolated as much as possible from orchards with other sources of blight resistance or trees at other stages of breeding. Likewise, seed orchards, such as of B_3-F_2 trees, are isolated as much as possible from other orchards. A distance between orchards of about 1 kilometer is estimated to be sufficient to isolate orchards. Pollen from undesired trees also is eliminated by emasculation, pruning at ground level and removal of the undesired trees.

Cultivation

The cultivation methods employed are standard orchard practices adapted to screening chestnut trees for blight resistance. Hebard (1991) discussed locating flowering American chestnut trees, and Hebard and Rutter (1991) outlined cultivation methods suitable for breeding orchards. Hebard (1994a) described the techniques for inoculating chestnut trees to test their blight resistance, and the orchard spacings used to grow trees. More recently, Hebard 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).

Orchards where backcross progenies are to be screened for blight resistance are arranged in completely randomized designs with controls consisting of 6 to 12 individuals each of pure American and pure Chinese chestnut trees, and their F_1 hybrid. This experimental design was chosen because each genotype is unique, with no replication of genotypes.

In a test of the response of trees of various ages to direct inoculation, the intermediate blight resistance of F_1 hybrids as young as 1 year old was distinguished from the high resistance of pure Chinese and from susceptible pure American chestnut trees. However, F_1 hybrids did not survive the test unless they were at least 3 years of age. Thus straight second backcrosses, which also have blight resistance up to the intermediate level found in F_1 hybrids, are screened for blight resistance when they are 3 or 4 years old. At those ages and under our growing condition, their diameter at breast height (1.5 m) ranges from 3 to 7.5 cm (1 to 3 inches) and their height from 3 to 5 m (10 to 15 feet).

In order to avoid crowding prior to blight resistance screening, trees to be screened at 3 years of age are grown at a spacing of 1.2 m (4 feet) within rows. Trees screened for blight resistance at 4 years of age are grown at a spacing of 2.1 m (7 feet) within rows. Originally, straight backcross trees were screened for blight resistance at 4 years of age. Currently, straight backcross trees are screened for blight resistance when they are 3 years old, except for third backcross trees, which are screened when 4 years old (we did not wish to change methods for our most valuable breeding material). Progeny of large, surviving American chestnut trees also are screened for blight resistance when they are 4 years old. To provide access for equipment, the between-row spacing in these orchards is 6 m (20 feet).

Progenies expected to contain blight-resistant individuals, such as F_2 generations, are screened for blight resistance when they are 1 or 2 years old. The blight-resistant progeny generally survive inoculation at that young age. These are spaced within rows at 30 or 60 cm (1 or 2 feet). The between-row spacing for F_2 progeny varies from 2.1 to 6 m (7 to 20 feet) depending upon the location and intent of the test.

Nuts are sown directly at orchard spacing. Prior to planting, orchard rows are subsoiled, plowed and rototilled, and 31.75-µm (1.25-mil) black plastic mulch lain in 1.22-m-wide (4 feet) strips. Using handled bulb planters, holes are drilled through the mulch into the soil and filled with a mix of one-third each ground, milled peat moss, perlite and coarse vermiculite. Nuts are planted 1-cm deep (0.5 inches) and protected from voles with aluminum cylinders 25.4-cm tall (10 inches) and 5 to 7 cm wide (2 to 3 inches). After planting, the cylinders are jammed down around the nuts to a depth of about 5 cm (2 inches). The

aluminum is painted to reduce aluminum toxicity should it dissolve into the soil. Soil is mounded around the cylinders to prevent them from being blown away by wind. Styrofoam cups are inverted over cylinders until shoots emerge from the cylinders. At that point, the bottom of the cup is removed, and the cup replaced, to diminish breaking of the young shoots on the edge of the cylinders.

The seedlings generally outgrow the width of the cylinders during their third growing season. At the beginning of the third growing season, the cylinders are removed. The mulch also is removed to reduce vole damage. Prior to this time, the cylinders prevent vole damage. Voles can be harbored under mulch.

While black plastic mulch is in place, trees are fertilized with soluble fertilizer with a major nutrient composition of 30-10-10 (N-P-K) plus cationic trace elements (MirAcid™ or equivalent). Liquid fertilizer is used in order to place the fertilizer under the impermeable mulch. Approximately 2 liters (2 quarts) of fertilizer solution is applied every 2 weeks between mid May and early August. The fertilizer concentration is 3.26 ml per liter (1.25 tablespoons per gallon of water). Fertilizer is pumped directly down the cylinders or applied through a drip irrigation system. Once plastic mulch is removed, granular fertilizer is broadcast around the trees. The rate for granular fertilizer usually is 224 kg per hectare (200 lbs per acre) of N as ammonium nitrate and diammonium phosphate, 67 kg per hectare (60 lbs per acre) of P as diammonium phosphate and 67 kg per hectare of K as potash. These amounts are applied twice a year, in mid May and late June. In seed orchards, to avoid having to apply liquid fertilizer underneath plastic mulch, landscape fabric is used for mulching and granular fertilizer is broadcast at the above rates. The rates were formulated from soil and foliar mineral analysis for the soils typical of Meadowview and might differ on other soils. The rates also are adjusted depending upon the results of soil mineral analysis.

On trees 5 years of age and younger, weeds are managed with herbicides and mulch. In general, no weed management is performed on trees older than 5 years of age, other than mowing. Currently, in April, Surflan™ A.S. (oryzlin) is applied at 9.35 liters per hectare (4 quarts per acre), simizine 4L at 7.02 liters per hectare (3 quarts per acre) and Roundup Ultra™ (glyphosate) at 3.07 liters per hectare (42 oz per acre). A supplemental spray of Roundup Ultra™ at 3.07 liters per hectare (42 oz per acre) is applied in July to trees younger than 3 years old. These herbicides are applied as a directed spray using TeeJet™ 8005 standard flat-fan nozzles operated at 2.07 bars (30 psi) in a water solution of 608 liters per hectare (65 gallons per acre). The combination of low pressure with high volume spray nozzles increases droplet size, reducing drift. A strip 152.4 cm wide (3 nozzles at 50.8-cm or 20-inch spacing, 45.72 cm or 18 inches above the ground) is sprayed down each side of a row. The nozzle closest to the trees is directed with a hand wand, the other two nozzles are mounted on the boom of the spray rig.

Grass strips are maintained between rows to reduce erosion. Fire hazard is reduced by regular mowing with rotary cutters. In B_3-F_2 seedling seed orchards, which are sown at much higher densities (0.3 x 2.1 m, 1 x 7 feet), maintenance is performed with a riding lawn mower. Weeding of seedling seed orchards is done as above, but using a 25-gallon tow-behind sprayer attached to the lawn mower rather than a 65-gallon herbicide spray rig mounted on the three-point hitch of the standard orchard tractors used in the larger orchards. Only two nozzles are used in seedling seed orchards. The lawn mower-mounted nozzle is attached to the front of the mower. The mower operator also can manipulate a hand wand fairly easily on the lawn mower, whereas on the larger orchard tractors it is best if the hand nozzle is operated by a person walking behind. A pressure regulator needs to be added to most tow-behind sprayers. Their pumps are driven by electric motors powered from the lawn mower's electrical system, whereas the power take off drives the pumps on the orchard tractors. Thus it is important that the lawn mower produce enough electric current to power the pump.

Using an airblast sprayer, aphids are controlled with a single application of dormant oil during bud break at 56 liters per hectare (6 gallons per acre) in 2807 liters per hectare (300 gallons per acre) of water

solution. In July, Japanese beetles are controlled with 2 to 3 applications of Sevin XLR Plus™ at 5.8 liters per hectare (0.625 gallons per acre) in 935 liters per acre (100 gallons per acre) of water solution. Spray amounts have been reduced considerably by employing a Durand-Wayland Smart Spray 1000™ attached to a Durand-Wayland model AF500CPS airblast sprayer. This device cuts off banks of nozzles depending upon tree height and occurrence.

The pesticide application methods, composition, and rates were formulated in consultation with extension specialists from the Virginia Polytechnic Institute and State University and the "Spray Bulletin for Commercial Fruit Growers," which is issued annually (Virginia, West Virginia & Maryland Cooperative Extension Services, 2004).

Straight backcross trees have been irrigated in the year of inoculation during dry years. Since the year 2000, all young chestnut trees have been irrigated, except B_3-F_2 seedlings, using a drip irrigation system. Soil moisture is maintained at field capacity (about 10-20 kiloPascals of soil moisture deficit). We plqn to not irrigate B_3-F_2 seedling seed orchards.

Trees are not pruned for shaping or for removal of lower branches, as is often done in commercial fruit and nut orchards to facilitate passage down the rows and weeding with herbicides, among other objectives. Not pruning results in a crown that extends to the ground on the trees (and necessitates a second person walking behind the herbicide sprayer to prune off portions of branches that are sprayed inadvertently). This larger crown may promote early and heavier bearing. For the most part, our trees produce male catkins when they are 2 to 4 years old and bisexual catkins when they are 3 to 5 years old. This early flowering also has been seen in other hardwood trees grown under intense cultivation (Wright, 1976).

Using the above methods, the trees at Meadowview have averaged 0.56 m tall after one growing season, 1.5 m (5 feet) tall after two, 2.4 m (8 feet) after three, and 3.7 m (12 feet) after four growing seasons. There can be considerable variation in height growth within orchards and between growing season, genotype and location.

Screening for Blight Resistance

The cork-borer, agar-disk method is used to inoculate chestnut trees with the blight fungus (Griffin, *et al,* 1983). Agar disks are obtained from the margins of growing cultures that have not reached the edge of the Petri plate. Inoculations are performed in early June. This is the earliest in the season when cool weather (daily high temperatures below 15 to 20 C) can be avoided reliably. Cool weather occurs every few years in late May in Meadowview and can lead to inoculation failure.

Two strains of the blight fungus are used, known as Ep155 and SG1 2-3. Ep155 is a widely used strain of the blight fungus (ATCC 38755), while SG1 2-3 was isolated near Meadowview by the author. When tested for pathogenicity in American chestnut, the distribution of lengths of cankers incited by virulent strains of the blight fungus follows a bell-shaped curve; it is approximately normally distributed, and variances are equal for the various canker lengths (Griffin, et al, 1983). When replicated five times each over 3 years, or 15 total replicates, Ep 155 was among the most pathogenic of 21 tested virulent strains, having significantly ($p < 0.05$) larger cankers than six of the least pathogenic test strains. Likewise, SG1 2-3 was among the least pathogenic of the 21 tested strains, having significantly smaller cankers than seven of the most pathogenic test strains.

Blight resistance can be determined quantitatively by measuring the length and width of cankers. Canker depth or superficiality is not determined at Meadowview since the intermediate to very high levels of blight resistance being sought can be distinguished using length and width measurements alone. Until

1999, the length and width of cankers was measured on all tested trees. Because this was taking too much time, beginning in 1999, blight resistance in most tests was determined using a qualitative assessment.

The qualitative assessment is based on the following observations. In general, 1 year after inoculation, SG1 2-3 incites small cankers (2-3 cm long) on trees with intermediate levels of blight resistance or higher. It incites medium-sized cankers (3-6 cm long) on trees with low levels of blight resistance, and large cankers (> 6 cm long) on normal American chestnut trees. In contrast, Ep 155 incites large cankers on trees with intermediate levels of blight resistance or less, medium-sized cankers on trees with high levels of blight resistance, and small cankers on trees with very high levels of blight resistance. Thus five blight resistance classes can be distinguished on trees inoculated with both strains. This is depicted visually in Table 3.

Table 3. Blight resistance classes distinguished qualitatively by various canker length classes for two strains of *Cryphonectria parasitica* one year after inoculation in early June.

Numeric blight resistance class	Verbal blight resistance class	Length (cm) of canker incited by	
		Ep 155	SG1 2-3
1	highly blight resistant	2-3	2-3
2	blight resistant	3-6	2-3
3	intermediately blight resistant	> 6	2-3
4	slightly blight resistant	>> 6	3-6
5	not blight resistant or susceptible	>>> 6	>6

Table 3 depicts idealized canker lengths for various blight resistance classes seen in average years. Depending upon the season, slightly blight-resistant trees might show small SG1 2-3 cankers or blight-resistant trees might show large Ep 155 cankers. Additionally, the responses to the two strains do not always move in parallel with each other. These various unusual patterns of response can be detected by the response of the pure American and Chinese chestnut trees and their F_1 hybrids planted as control trees in the orchard and the scale adjusted accordingly.

In addition to artificial inoculation, trees in Meadowview also are exposed to naturally occurring inoculum. Blight incidence due to natural infections on straight backcross progeny exceeds 50% by the beginning of the fifth growing season, when trees are four years old. When screening artificially inoculated trees for blight resistance, the severity of these naturally occurring cankers is considered in the overall assessment of a tree. Thus, while only two strains of the blight fungus are used for direct inoculation, a larger number of strains is involved in the overall assessment.

RESULTS AND DISCUSSION

Recovery of highly blight-resistant backcross progeny at F_2

The first screening of progeny segregating for blight resistance in Meadowview occurred in 1993. One set of progeny consisted of B_1-F_2s obtained from reciprocal crosses of the 'Graves' and 'Clapper' trees. A second set of progeny consisted of straight F_2s obtained from a one-way cross of two F_1s. The F_1

parents were half sibs from crosses of the 'Mahogany' Chinese chestnut tree with pollen from two American chestnut trees. A third set of progeny segregating for blight resistance consisted of straight B_2s composited from three crosses of pollen from the 'Graves' tree onto three American chestnut trees. The trees were 2 years old when inoculated in June, 1993, and the data in Table 4 summarize canker dimensions when measured in September, 1993. Each tree was inoculated once with strain Ep 155 and once with strain SG1 2-3, using the cork borer, agar-disk method with holes 2 mm in diameter. Highly blight-resistant progeny were recovered from the F_2 and the B_1-F_2 crosses, and progeny with intermediate levels of blight resistance were recovered from the B_2 crosses. The B_1-F_2 crosses may have had higher blight resistance than the straight F_2s. Figure 1 depicts one of these highly blight-resistant B_1-F_2s.

Table 4. Mean and standard deviation and distribution of canker size classes (mean length and width of cankers incited by two strains of the blight fungus) for straight F_2, B_1-F_2 and B_2 American x Chinese chestnut progeny and controls.

Cross Type	Canker size class (cm)							Mean	Standard deviation
	1.0 to 2.6	2.6 to 4.2	4.2 to 5.8	5.8 to 7.4	7.4 to 9.0	9.0 to 10.6	10.6 to…		
Seedling American					3	5	2	9.6	1.1
F_1 'Nanking'				2	4	3		8.4	1.0
Seedling Chinese		2	7	3				5.2	1.0
'Meiling' Chinese		1	2	2				5.5	1.1
'Nanking' Chinese	3		2					2.9	1.4
F_2 'Mahogany'		5	23	48	48	29	15	7.7	1.9
B_1-F_2 'Clapper' x 'Graves'	4	25	84	116	112	54	4	6.9	1.9
B_2 'Graves'			2	4	15	26	6	9.1	1.5

Three-year-old B_2-F_2 progenies from controlled crosses between selected straight B_2s (backcrossed to American chestnut) were inoculated in June, 2003, and cankers measured in November. 'Clapper' B_2-F_2 progeny were from a single cross between two half sibs, while 'Graves' B_2-F_2 progeny were a composite of three crosses between half sibs. Depending upon their size, these trees were inoculated once or twice each with strains Ep 155 and SG1 2-3, using the cork borer, agar-disk method, but the holes were 4 mm in diameter. A larger cork borer and number of inoculations were used in 2003 than in 1993 because 2003's 3-year-old trees were larger than 1993's 2-year-old trees. Again, highly blight-resistant progeny were recovered, this time from second backcross F_2s (Table 5). Thus, not only could highly blight-resistant progeny be recovered by intercrossing F_1 interspecific hybrids or by intercrossing first or second backcrosses to American chestnut, but high levels of blight resistance were retained through the second backcross. These results suggest very strongly that the blight resistance of Chinese chestnut can be backcrossed into American chestnut.

Canker sizes were smaller in the 2003 than in the 1993 test, possibly because of cooler, wetter weather in the later year, so there was not as much separation of canker sizes among the controls. However, the cankers on some of the B_3-F_2 progeny have remained small through the 2004 growing season, as illustrated in Figure 2. An earlier test, performed in 1999 on open-pollinated progeny of 'Clapper' B_2s,

Figure 1. Highly blight-resistant Chinese to American B1-F2, 13 years old, 11 years after inoculation with *Cryphonectria parasitica.* The tree is to the left of and behind the dog.

presumably pollinated by other 'Clapper' B_2s, gave results similar to those presented in Table 5 (Hebard *et al*, 2000).

Blight resistance in straight backcrosses

Tables 6, 7, and 8 report typical results of rating straight second and third backcross trees for blight resistance. An entire family derived from a second backcross tree has not yet been rejected based on the performance of its third backcross progeny. In general, the blight resistance of third backcross progeny is comparable to that observed in second backcross trees, again supporting the inference that there is no diminution of resistance as backcrossing proceeds.

Family effects have occurred in second backcross progeny fathered by both the 'Graves' and 'Clapper' trees, where the American mother of second backcross progeny influenced their phenotypic blight resistance. This is illustrated in Table 9, where the Bu3C1C x 'Clapper' family had cankers closer in size to cankers on Chinese chestnut than on F_1s or Americans. It is unclear whether or not the Bu3C1C American parent was contributing genes for blight resistance by itself or contributing genes that

Table 5. Distribution of canker size classes (mean length and width of cankers incited by two strains of the blight fungus) for B_2-F_2 American x Chinese chestnut progeny and controls.

Cross Type	Canker size class (cm)							Mean	Standard deviation
	1.0 to 2.0	2.0 to 3.0	3.0 to 4.0	4.0 to 5.0	5.0 to 6.0	6.0 to 7.0	7.0 to 8.0		
Seedling American			4	2	2	2	1	5.0	1.4
F_1 'Nanking'		1	2	3	1			4.1	1.0
Seedling Chinese	3	3	3	6				3.3	1.2
B_2-F_2 'Clapper'	3	11	15	37	16	12	3	4.5	1.4
B_2-F_2 'Graves'	3	11	21	31	14	14	1	4.4	1.3

Table 6. Blight resistance ratings of 'Clapper' and 'Graves' second backcross trees and controls in 1999.

Cross type	Blight resistance rating				
	1	2	3	4	5
Seedling American				2	3
F_1 'Nanking'		4			
Seedling Chinese	3	5			
'Nanking' Chinese	1	1			
B_2 'Clapper'		5	27	29	12
B_2 'Graves'		3	42	47	25

Table 7. Blight resistance ratings of 'Clapper' third backcross trees and controls in 2000.

Cross type	Blight resistance rating				
	1	2	3	4	5
Seedling American				3	3
F_1 'Nanking'		2	10		
Seedling Chinese	3	2	1		
B_3 'Clapper'	1	19	139	383	95

Table 8. Blight resistance ratings of 'Graves' third backcross trees and controls in 2001.

Cross type	Blight resistance rating				
	1	2	3	4	5
Seedling American			1	3	8
F_1 'Nanking'		2	5		
Seedling Chinese	7	8			
B_3 'Graves'			124	124	122

modulated the expression of blight resistance genes from Chinese chestnut. The Bu3C1C tree itself did not appear to have more blight resistance than typical American chestnut trees; it died from blight the year after this cross was made, like most of the other American chestnut trees at that site.

Figure 2. Left, chestnut blight cankers after two growing seasons on a highly blight-resistant 'Clapper' B_2-F_2. Top left, canker incited by strain SG1 2-3. Bottom left, canker incited by strain Ep 155. Right, 4-year-old 'Clapper' B_2-F_2. Similar cankers on blight-susceptible American chestnut would be expected to exceed 40 cm in length; these cankers were 2 to 3 cm long.

Table 9. Distribution of canker size classes (mean length and width of cankers incited by two strains of the blight fungus) for progeny of second backcrosses of the 'Graves' and 'Clapper' first backcross trees to American chestnut and controls, in 1998.

Cross type	Canker size class (cm)						
	2 to 4	4 to 6	6 to 8	8 to 10	10 to 12	12 to 14	14 to 16
Seedling American					4	1	
F_1 'Nanking'			1	3	1		
Seedling Chinese		3	4				
'Nanking' Chinese	2	2					
Bu2B2C x 'Clapper'			2		3	2	
Bu2B3C x 'Clapper'			3	4	4	1	
Bu3C1C x 'Clapper'		15	33	8			
Bu1C1G x 'Graves'			4	8	17	10	1
Bu1C2G x 'Graves'			2	1	1	1	
Bu3B1G x 'Graves'				1			
Bu3B2G x 'Graves'					2		
Bu3C3C x 'Graves'		4	8	15	25	19	2
Bu3D1G x 'Graves'			1		2		
Bu3F1G x 'Graves'					1	1	
Bu3F5G x 'Graves'			2	2	5	2	
Bu3R1G x 'Graves'			2	7	13	4	1

Number of genes conditioning blight resistance

The standard deviations of canker size in Table 4 were greater for the progeny expected to be segregating for blight resistance than for the controls, and, for the F_2s, were compatible with models for one or two incompletely dominant genes controlling blight resistance, using Wright's method for estimating the number of factors controlling a segregating trait (Falconer, 1960, p 218). (In this computation, the total genetic variance of the F_2s was substituted for the additive genetic variance; the former was computed by subtracting the mean variance of the controls from the variance of the F_2s. The broad sense heritability calculated from these variances was about 70%). The distributions of canker size in segregating progeny in Table 4 were compatible with the distributions of canker size expected for two or three incompletely dominant genes of equal effect on blight resistance, among other models for gene action. Similar models with more than three factors or fewer than two did not fit the observed values (chi-square $p<<0.05$). The expected distributions were constructed from the mean response for the control trees, assuming a normal distribution of canker size with the average standard deviation of the controls shown in Table 4; missing cells, such as for trees with only one allele for resistance, were estimated by linear interpolation between the relevant observed values. Unfortunately, vegetatively propagated (grafted) individuals of 'Mahogany' were not available for inclusion in the test, nor the actual F_1 parents; otherwise stronger inferences might have been possible concerning the mode of inheritance of blight resistance. Subsequent experience suggests that 'Mahogany' has a high level of blight resistance, comparable to that of 'Nanking.' This

suggests in turn that two genes are involved in blight resistance. A three-gene model would be more compatible with the data if 'Mahogany' Chinese chestnut had a "normal" level of blight resistance like the 'Meiling' and seedling Chinese in Table 4 rather than the high level of blight resistance observed in 'Nanking.'

Kubisiak, *et al.* (1997) prepared a genetic map of the 'Mahogany' F_2s whose canker sizes are shown in Table 4. Their results indicated that three regions of the genome were associated with blight resistance. The Kubisiak, *et al.* (1997) map was constructed with randomly amplified polymorphic deoxyribonucleic acid markers (RAPDs), restriction fragment length polymorphic markers (RFLPs), and isozymes. Subsequent genotyping of the mapping population with markers based on simple sequence repeats (SSRs) indicated that 17 of the 185 progeny were outcrosses, not pollinated by the supposed male parent (Sisco, Kubisiak and Hebard, unpublished). These individuals are not included in Table 4. One of the three regions of the genome previously associated with blight resistance (located on Kubisiak *et al.* (1997)'s linkage group G) was no longer associated with blight resistance in the revised mapping population. Molecular mapping of backcrosses of 'Mahogany' F_1s to American chestnut also suggested that the same two regions of the genome condition blight resistance (Kubisiak and Hebard, unpublished). The molecular mapping data thus supported a model of two incompletely dominant genes conditioning blight resistance in these progeny.

Highly blight resistant 'Clapper' x 'Graves' B_1-F_2 individuals were test crossed to American chestnut to determine whether or not they were homozygous for blight resistance. Screening of these 'Clapper' x 'Graves' test cross progenies indicated that they were segregating for blight resistance (data not shown), and hence that the B_1-F_2 parents were not homozygous. This finding suggests that some of the genes conditioning blight resistance in 'Clapper' and 'Graves' are at different loci. Highly blight-resistant 'Mahogany' F_2 progeny also had been test crossed to American. Unfortunately, all of the test-crossed individuals turned out to be outcrosses, as indicated by the SSR markers, invalidating this second set of tests.

There are numerous patterns of inheritance possible when a trait is controlled by more than one gene, including complementary inheritance, epistasis, etc (Grant, 1975). The model here of two incompletely dominant genes of equal effect is only one among these models, albeit one that fits the data. If further improvement of backcross chestnut trees for blight resistance is necessary beyond the B_3-F_2 stage of breeding, it might be best to use breeding methods for quantitative traits, such as recurrent selection.

The fact that the variance or range of canker sizes for the F_1 controls in Tables 4 to 9 were similar to those of the pure species indicates that 'Nanking' Chinese chestnut trees are homozygous for blight resistance. Similar data suggest that the named Chinese chestnut cultivars Orrin and Meiling, and the unnamed cultivars of Greg Miller, 64-4 and 72-211, likewise are homozygous for blight resistance.

Outbreeding and inbreeding depression

Not infrequently, specific Chinese x American chestnut crosses fail to produce nuts. Sometimes, nuts are produced, but fail to grow after germinating a radicle. These failures may be considered extreme instances of outbreeding depression. Chinese x American F_1 hybrids that do germinate often exceed pure species in size up to 10 to 20 years after planting, exhibiting hybrid vigor. For instance, after three seasons of growth, F_1 hybrids in four orchards were significantly ($p < 0.0001$) taller than pure species, having a least squares mean height of 2.2 m versus 1.8 m for the pure species. The F_1 hybrids also were significantly taller than any of the individual pure species.

The 'Mahogany' F_2s of Table 4 came from the only intercross of Chinese x American F_1 hybrids that has yielded well (greater than 1.0 nuts per pollination bag). Other F_1 intercrosses have yielded fewer than 0.6

nuts per pollination bag, sometimes much less. Attempts to use Chinese x American F_1 hybrids to pollinate American or Chinese chestnut trees also have produced low yields, in general. Even some intercrosses among half-sib B_2s have yielded sound nuts that failed to produce seedlings. The failures of some of these more advanced crosses may be due to inbreeding depression rather than outbreeding depression. The failures (and pollen contamination in our early crosses) bedeviled attempts to repeat the early experiments. Similar failures also may have bedeviled attempts of earlier researchers to test hypotheses regarding the inheritance of chestnut blight resistance.

As mentioned previously in the section on blight resistance, the 'Clapper' x 'Graves' B_1-F_2s of Table 4 had more apparent blight resistance than the Mahogany F_2s. They also grew to be larger, more vigorous trees, perhaps because they did not suffer from inbreeding depression and/or had hybrid vigor (four-hundred, nineteen 'Clapper x Graves' and 'Graves x Clapper' progeny had a mean height at the end of the 1993 growing season of 2.43 m while 191 'Mahogany' F2s had a mean height of 2.13 m, significantly shorter, $p<0.0001$; a similar trend, $p=0.001$, was observed in 1992, prior to inoculation). The relative contributions of general vigor versus specific genes for blight resistance to the greater phenotypic blight resistance of the 'Clapper' x 'Graves' B_1-F_2s are unclear.

Summary

In sum, we have been able to recover highly blight-resistant chestnut trees after backcrossing blight resistance from Chinese into American chestnut for two cycles of backcrossing. Three cycles of backcrossing are expected to produce chestnut trees that, for the most part, look and grow like American chestnut. We currently are starting to test the blight resistance of second-generation, third backcross trees (B3-F2s), and currently expect some of them to have high levels of blight resistance. By 2008, we hope to begin planting their progeny (B3=F3s) back into the forest to confirm these expectations and to begin restoring the American chestnut tree to Appalachian forests.

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