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SELECTION FOR CHINESE VS. AMERICAN GENETIC MATERIAL

IN BLIGHT RESISTANT BACKCROSS PROGENY USING GENOMIC DNA

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Abstract: The American chestnut (*Castanea dentata* [Marshall] Borkhausen) was historically one of the most important hardwoods in North America due to its abundance and the multiple functions it served for both ecosystems and humans. The exotic chestnut blight fungus has eliminated the American chestnut as an overstory tree in eastern forest ecosystems, however. Backcross breeding shows promise to produce chestnuts that combine the blight-resistance that evolved in Chinese chestnut (*C. mollissima* Blume) with the desirable characteristics of American chestnut as a forest tree. In the backcross program, blight-resistance is introduced by an interspecific cross of American chestnut with resistant Chinese chestnut trees. American chestnut characteristics are then regained by a series of backcrosses to American chestnut parents. To accelerate and improve this selection process, we developed a molecular protocol to determine the amounts of American vs Chinese chestnut genome among progeny selected for blight resistance. The dot blot technique involves the hybridization of labeled Chinese chestnut genomic DNA to DNA from individual backcross progeny trees, which reveals the amount of Chinese chestnut DNA that remains in them. On average, progeny in the third backcross (BC3) generation should show lesser amounts of hybridization to Chinese chestnut genomic DNA probe than F2, BC1 and BC2 progeny. Because there will be variation among individuals in each backcross generation for the amount of Chinese chestnut genome that they contain, those blight resistant progeny with greater amounts of Chinese chestnut content can be identified by this approach and eliminated from the crossing program. The effectiveness and reliability of this approach are demonstrated using samples from the parents and progeny in three backcross generations.

Keywords: Backcross generations, dot-blot hybridization , genomic DNA, selection

INTRODUCTION

American Chestnut and the Chestnut Blight

Before the introduction of the chestnut blight disease, the American chestnut (*Castanea dentata* [Marshall] Borkhausen) was one of the most important trees in hardwood forests of the eastern United States. With a range centered on the Appalachian Mountains and extending from Maine west to Michigan and south to Alabama and Mississippi (Little 1976), the American chestnut grew in mixtures with many other species, and often comprised 25 percent or more of the hardwood tree population within any given forest stand (Braun 1950).

The American chestnut may have been the most important hardwood in eastern North America due to its abundance and the multiple functions it served for both ecosystems and humans (Hardin *et al*. 2001). It was a dominant component of much of the eastern hardwood forest, and it produced a regular and bountiful nut crop that was an important part of the diet of many animals (Rice *et al.* 1980). Historically, the American chestnut was an important tree because of the assortment of services and commodities it provided to people as well. It was an extraordinary tree for wood fiber production due to its large size, fast growth, and ability to sprout from stumps (Detwiler 1915). American chestnut wood fulfilled a multitude of needs ranging from construction and furniture lumber, firewood, fence construction, railroad ties, telephone and telegraph poles, pulpwood, and tannins. The chestnuts were also important as a food source for rural residents, and the tree was widely planted to provide shade (Buttrick 1915).

Chestnut blight was first introduced to North America in 1904. The chestnut blight disease is caused by *Cryphonectria parasitica* (Murrill) Barr (=*Endothia parasitica* [Murrill] P.J. and H.W. Anderson), an exotic fungus from Asia that enters through wounds in the bark and eventually girdles the tree, killing susceptible individuals (Roane *et al.* 1986). Because American chestnut trees evolved in the absence of the fungus, they lacked entirely any genetic protection from the fungus (Stiles and Hebard 1996). By 1950 the disease had spread across the entire native range of the American chestnut, eliminating it as an overstory tree in eastern ecosystems (Newhouse 1990). The American chestnut continues to survive as a shrub, however, sprouting from the root collars of stumps in the forest (Hardin *et al*. 2001).

Backcross Breeding Program

The American Chestnut Foundation's (TACF) approach to developing the most resistant trees with the best American characteristics – "*the path of most resistance*" – is shown in Figure 1. After the chestnut blight fungus was introduced to the United States, plant explorer Frank Meyer discovered the fungus in Asia, along with Chinese chestnuts (*C. mollissima* Blume) that had evolved resistance to the disease (Fairchild 1913). Because of the blight resistance of Chinese chestnut, and cold hardiness, this species was selected for developing blight-resistant hybrids with American chestnut that could replace the disappearing (Burnham 1987) in American forests.

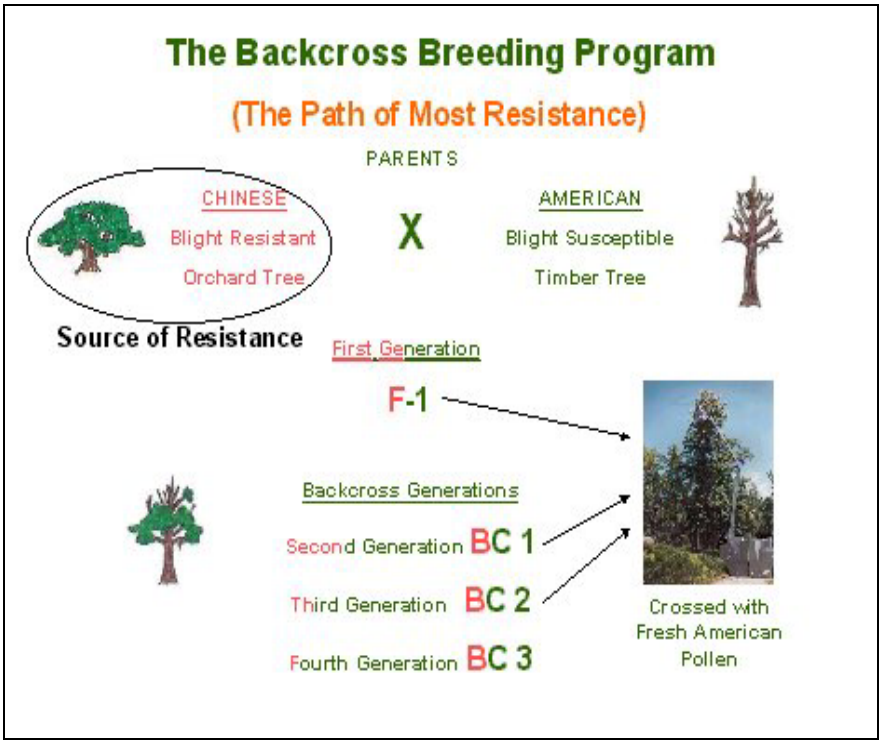


Figure 1. The scheme for the backcross breeding program being used by the American Chestnut Foundation (from Hebard, Http:// chestnut.acf.org).

However, while Chinese chestnut is highly resistant to the chestnut blight, it has other characteristics that make it undesirable as a replacement for the American chestnut. Whereas the American chestnut grows straight and tall and was formerly a canopy tree species, the Chinese chestnut has a low-growing, sprawling form similar to that of an apple tree. Additionally, American chestnut trees have

higher quality timber, sweeter nuts, and a faster growth rate (Hebard 1994a; Stiles and Hebard 1996). The genetic material of the American chestnut also reflects thousands of years of co-evolution with eastern hardwood forest ecosystems. During this time, complex relationships presumably evolved between the American chestnut and other components of the forest, a history that is borne in the genome of the American chestnut (Stiles and Hebard 1996). Thus a program based on back-cross breeding (Figure 1) was developed to recover the American characteristics while retaining the Chinese blight resistant genes.

RATIONALE AND APPROACH

The process of recovering the American characteristics by diluting out all of the Chinese donor parent characteristics, except for blight resistance, usually entails several generations of backcross breeding to recurrent parent trees (AC). The first hybrid generation (F_1) produced by crossing American chestnut with Chinese chestnut inherits one half of its genes from the American chestnut parent and one half from the Chinese parent. These first-generation hybrids are then backcrossed to an American chestnut parent, producing a first backcross generation (BC_1) that has a genome that is on average three-quarters American chestnut and one-quarter Chinese chestnut. Each successive backcross reduces the Chinese fraction of the genome by one-half: the second backcross generation (BC_2) is on average one-eighth Chinese chestnut, and the third and final (in the plan outlined by Burnham) backcross generation (BC_3) is on average fifteen-sixteenths American chestnut and one-sixteenth Chinese chestnut (Rutter and Burnham 1982). However variation occurs among individuals in each backcross generation for the amount of Chinese chestnut genome that they contain due to chromosomal recombinations that naturally occur at gamete formation. In addition, TACF produces intercross (F2) generations (Figure 2) that increase the number of progeny at each generation, and provide greater genetic variation and greater opportunity for blight resistance to be separated from other tree characteristics. Selection for blight resistance and tree characters is made at each breeding step, which requires intervals of several years. The breeding program could be accelerated through

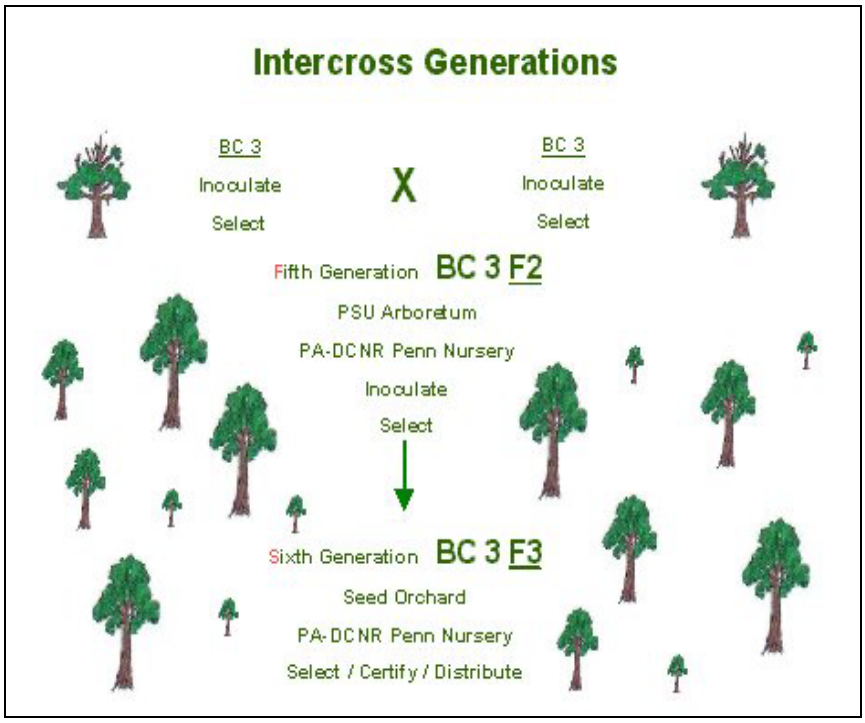


Figure 2. Advanced generation intercross scheme of the American Chestnut Foundation for seed orchard development and production (Http:// chestnut.acf.org)

the use of genomics tools for the identification of trees carrying larger portions of American genome at each step, thus also improving the results of each stage of selection.

Many decades of breeding research by the U.S. Department of Agriculture, the Connecticut Agricultural Experiment Station (CAES), and the American Chestnut Foundation indicate that resistance in the Chinese species is carried on two or three genes, which are only incompletely dominant. To achieve full resistance, all the genes from American chestnut that control response to the blight must be replaced by the Chinese alleles. The ACF breeding program has already reached the third backcross generation which is being evaluated in extensive field tests in several states for durability of resistance and for the American tall-timbered growth habit and regional adaptability. Overall, TACF has more than 11,000 trees at various stages of the blight resistance breeding process at its farms in Virginia.

Random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers have been used to construct genetic linkage maps and identify genomic regions (QTLs) conditioning resistance in an F2 population derived from the 'Mahogany' resistance source (Kubisiak et al. 1997). Two of theses regions have since been confirmed in a BC1 population derived from 'Nanking' and PI 34517 suggesting that some of the genomic regions conditioning resistance are syntenic across the different sources (Kubisiak, unpublished). AFLP markers were subsequently been found that flank the blight resistance QTLs and that could thus also be used to select for those loci in progeny (Sisco, unpublished).

However, while individual RAPD, RFLP, and AFLP markers will be good for early selection for the major resistance loci, such linked markers and associated maps with DNA markers will be difficult to use efficiently to select against the chromosomal material from Chinese chestnut that is not associated with resistance. To select against the Chinese genetic background, it will be necessary to use many markers covering all of the linkage maps simultaneously. When many markers are being used in concert, the inherent problems with reproducibility of RAPD markers, with dominance of the AFLP and RAPD markers, and with length of time and inconvenience needed to use RFLP markers would make selection against Chinese genetic background by the DNA marker approach very complicated, and quite expensive.

We have developed a simple dot blot protocol to rapidly screen individual trees in the breeding program for their content of American versus Chinese chestnut genome. The technique involves the hybridization of labeled Chinese chestnut DNA to the DNA of individual trees, using American chestnut DNA to block the detection of sequences shared by American and Chinese chestnut.

In the present study we tested the effectiveness and reliability of the dot blot technique to directly select against the Chinese genome in progeny of the BC3 generation. On average, progeny in the BC3 generation should show lesser amounts of hybridization to the Chinese chestnut genomic DNA probe than F2, BC1 and BC2 progeny. It should also be possible to identify those blight resistant progeny within each backcross generation with greater amounts of Chinese chestnut content by this approach, so that they can be eliminated from the crossing program.

The second objective of this study was to evaluate how closely the data from the dot blot protocol correlated with visual evaluation of known morphological characteristics. We assume that the variation of hybridization of American genomic DNA among individuals within each BC generation is coincident with the variation of the morphological characteristics that taxonomically distinguish American chestnut and Chinese chestnut. In the study of variation of the morphological characteristics among individuals within generations conducted by Matt Diskin (2003, and previous chapter in this proceedings), twenty-four morphometric characteristics known to discriminate between American and Chinese chestnut were measured on each of approximately 50 individuals in the parental species, the first-generation hybrids, and in each of the three backcross generations. Principal components analysis was used to develop an Index of Species Identity (ISI) that described the aggregate

morphology of the different populations. As expected, the morphologies of American and Chinese chestnut were the extremes measured in this study. The first-generation hybrids were intermediate between the two parental species, and the three backcross generations had similar morphologies, distinct from Chinese chestnut and largely similar to American chestnut. American chestnut morphology was essentially recovered in the third backcross generation, for the 24 characters studied. To find the relationship between variation in hybridization data and morphology, DNA was obtained from the sample individuals used in the morphology study.

MATERIALS AND METHODS

Chestnut Materials and Sample Selection

Tissue samples were collected by Matthew Diskin (undergraduate thesis, PSU, December 2003) from trees at The American Chestnut Foundation's Glenn C. Price Research Farm in Meadowview, Virginia. Samples were taken from representative American and Chinese chestnut parents trees, their first-generation hybrids, and first, second, and third generation backcross hybrids (Table 1.).

Table 1. Populations sampled for morphology and dot-blot studies.

Population	Plantation and year planted[1]	Years since planting	Sample size for ISI study	Sample size for dot blots
American	Amer 2001	2	50	10
Chinese	CbyCs 2000	3	49	10
F_1	More F1s 1997	6	50	10
BC_1	JB1s 1999	4	60	30
BC_2	JB1s 1999	4	45	26
BC_3	Ilas 2000	3	49	28

[1]The plantation name refers to the chestnut plots at The American Chestnut Foundation's Glenn C. Price Research Farm in Meadowview, Virginia.

The population of American chestnuts represented the open-pollinated progeny of seven chestnuts growing wild in Smyth County, Virginia. The population of Chinese chestnuts was composed of two unique pedigrees, derived from controlled pollinations between two different sets of Chinese parents. All American chestnut parents in the backcross generations were the plantation-grown progeny of open-pollinated trees growing wild in the mountains of Virginia, except that one was itself a tree growing wild. Neither the American nor Chinese chestnut parents that were sampled were used as parent trees in any of the hybrid crosses. Twelve pedigrees of first-generation hybrids were sampled. These trees were the progeny of nine Chinese chestnut mother trees and 12 American chestnut father trees.

The populations of first-generation backcross trees sampled were progeny of a single American chestnut tree crossed with a single first-generation hybrid tree. Three pedigrees composed the population of second-generation backcross trees. The same first-generation backcross tree was used in each pedigree, but a different American chestnut parent was used in each cross. The population of third-generation backcross trees measured for this study comprised the progeny of a single second-generation backcross tree and a single American chestnut tree. There were no Chinese or American parents in common between the first hybrid and any backcross generations or between the various backcross generations (see Hebard, this volume).

DNA Extraction, Digestion and Transfer

DNA was extracted from twig samples that were selected for DNA dot blot analysis from among 10 individuals among the chestnut parent and the first generation hybrid populations (Table 1). The samples were selected based on the Indices of Species Identity (ISI) determined by Diskin (2003) with the approximate ratio of 1:2. Thus, the selected samples from the 3 BC generations should have the same distribution and population coverage as the original set of twig samples used by Diskin. The sample sizes in the 3 BC generations used for DNA extraction were: 30 samples in BC1, 26 samples in BC2, and 28 samples in BC3.

DNA extraction followed the manufacturer's instructions (Qiagen DNAeasy kit). Methods for DNA restriction enzyme digestion, agarose gel electrophoresis and alkaline transfer of DNA to nylon membranes were as described by Sharp et al. (1988), with minor modifications such as the use of Hybond N+ membranes (Amersham). Total genomic DNA was digested to completion using HindIII restriction endonuclease (Gibco). The agarose gels were stained with ethidium bromide and only those gels in which all tracks of genomic DNA showed approximately equal amounts of DNA after UV photography were used for transfer.

DNA Quantification and DNA Dot Blot Preparation

The individual tree DNA samples were quantified with a GeneQuant (Amersham) spectrophotometer (A_{260}). All the DNA samples were diluted to 50ng/μL with ddH_2O. Methods for manual preparation of the DNA dot blots followed the protocol provided by the nylon membrane manufacturer (Amersham), except that 1 μL of 50ng/μL of denatured DNA sample was applied for each dot. In the simulation experiment, two repeated applications were applied to each dot, for a total of 100ng DNA. All the DNA samples were applied to the filters in a random order, following the random numbers generated by use of the MINITAB program (MINITAB 13.32, Minitab Inc. 2000). The applied ssDNA was fixed to the membranes using a UV crosslinker (Stratalinker®, Stratagene) for 30 sec.

Probe Labeling and Southern Hybridization

The labeling of probes with radioactive P-32, the hybridization methods and the detection of hybridization signals followed manufacturer's instructions (Amersham). Briefly, total genomic DNA was mechanically sheared by syringe, the length of probes was estimated by gel electrophoresis to be about 500bp. The probes were denatured by boiling for 5 min and then labeled with P-32 by following the random priming protocol (Invitrogen). The membrane was incubated at 65□ overnight in the prehybridization buffer with the denatured salmon sperm DNA.

For experiments involving genomic blocking DNA, DNA fragments of 100-200 bp length were obtained by autoclaving the total genomic DNA for 2 min. The required amount of blocking DNA, 1-10 μg mL^{-1}, was denatured by boiling for 10 min, added to the hybridization buffer surrounding the membrane and incubated at 65□ overnight. The labeled probe (10-20 ng mL^{-1}) was added and the incubation continued for 8-16 hr at 65□ in the hybridization incubator.

Washing and Signal Detection

After hybridization, weakly hybridized and unhybridized probe was removed by three washes of 30 min each in 1) 2 X SSC (20 X SSC: 3M sodium chloride, 0.3 M sodium citrate, pH7)/0.1% SDS (sodium dodecyl sulphate) at room temperature; 2) 0. 2 X SSC/0.1%SDS at 42□; 3) 0.1XSSC/0.1%SDS at 65□. Hybridization sites were detected using a phosphor imager after the membranes had been exposed to the imaging screen for 2 h.

Signal Normalization and Quantification

Probe hybridization was measured quantitatively with a microcomputer-based image digitizing system TotalLab 2.00(Nonlinear Dynamics Ltd., 1996-2000). The intensity of the signals was digitized (Figure 3), and each measurement was normalized to the values of the positive controls (Figure 4). To compare the digitalized signal data from each dot, normalization was used to equalize the volumes in the dot images. This was accomplished by setting the normalized volume of dots from a serial dilution to specific values (positive controls) and then recalculating all other volumes relative to those values.

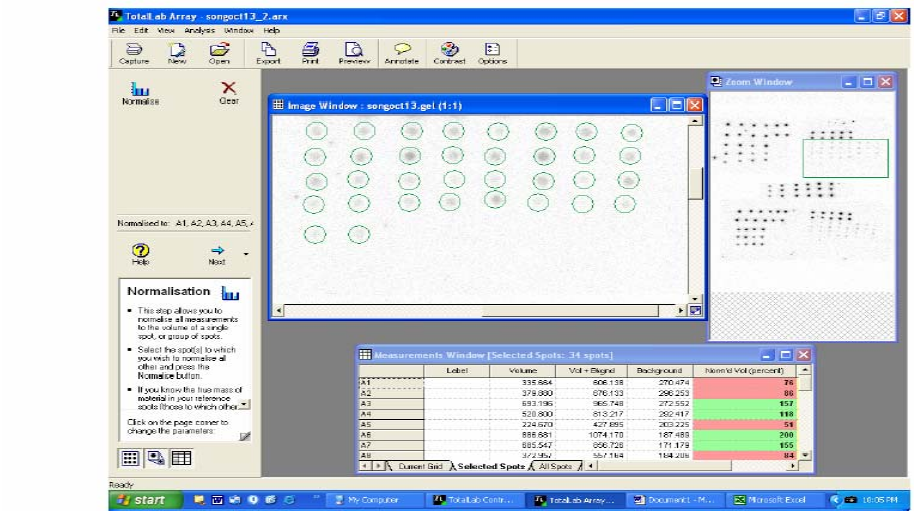


Figure 3. Screen capture of example signal quantification of dot blot using TotalLab 2.00.

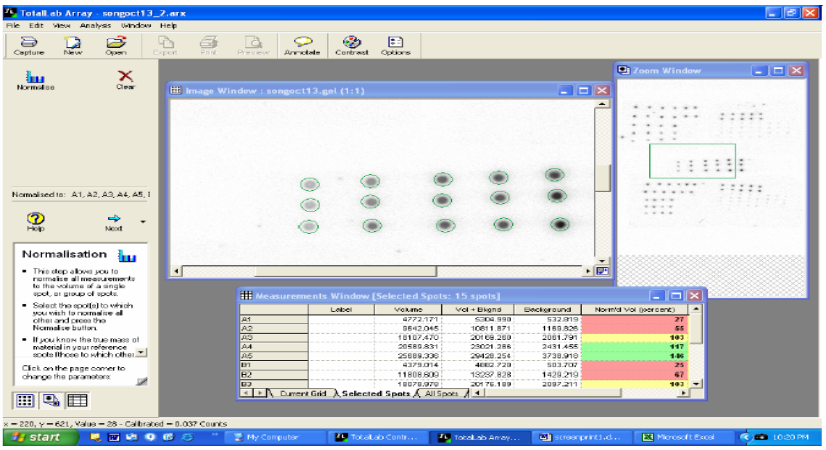


Figure 4. Screen capture of signal normalization by TotalLab 2.00 for positive control dilution example.

Statistical Analysis

Statistical analysis of the normalized dot blot signal data was performed using Minitab version 13.32 (Minitab Inc. 2000). Analysis of variance (ANOVA) was used to test the significance of mean differences within populations for signal intensity assuming equal variances. Brown and Forsythe's test was used to test for equal variance (Brown and Forsythe 1974). No data transformations were necessary. Pearson's correlation coefficient was calculated to find the relationship between morphometric data and hybridization data (Minitab Inc. 2000).

RESULTS

Differentiation of American Chestnut and Chinese Chestnut

Preliminary experiments were conducted with parental DNAs to test the effectiveness of unlabeled American chestnut genomic DNA in blocking hybridization signal from shared sequences in the Chinese chestnut probe. The autoradiogram in Figure 5 shows the hybridization intensities obtained with labeled genomic Chinese chestnut probe hybridized to Southern blots of HindIII digests of parental and backcross generation genomic DNAs after two low stringencies washes. In the left panel of Figure 5, with no blocking DNA used, strong probe hybridization to DNA tracks from all generations is visible, and bands of restriction fragments from highly repeated DNA families are of similar intensity among samples. When the membrane was blocked with unlabelled DNA from American chestnut (right panel of Figure 5), the hybridization of Chinese chestnut DNA probe to the American and BC3 samples were greatly decreased, while the amount of hybridization to the Chinese, BC1 and BC2 samples were decreased to a lesser extent. In addition, the intensity of hybridization for the smallest band in Figure 5 (arrow) is increased in the F1 and BC samples after blocking, while the American sample maintains the same low intensity, suggesting that this restriction fragment is Chinese –specific and when the American genomic DNA was blocked, the band was more accessible to the probe.

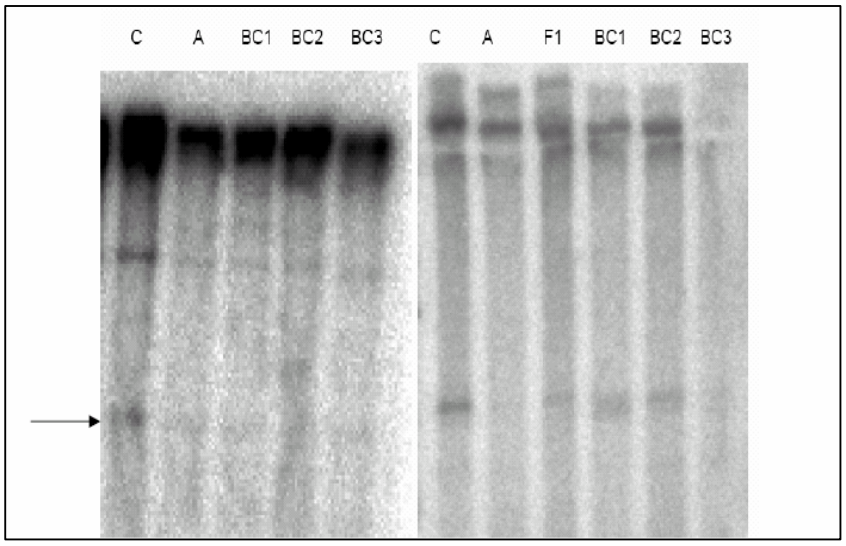


Figure 5. Southern Blot of genomic DNAs digested by HindIII, and hybridized against Chinese total DNA probe, labeled with P32. Left panel: No blocking DNA ; Right panel: American chestnut blocking DNA .

Signal Normalization With the Controls

In this project, we normalized signals within blots by using a serial dilution of known amounts of Chinese chestnut and American chestnut DNAs on the blots as positive controls (Figure 6). To avoid bias caused by experimental errors, the internal controls in each dot blot were used to normalize the dot signals among blots probed by Chinese total DNA, with American blocking DNA.

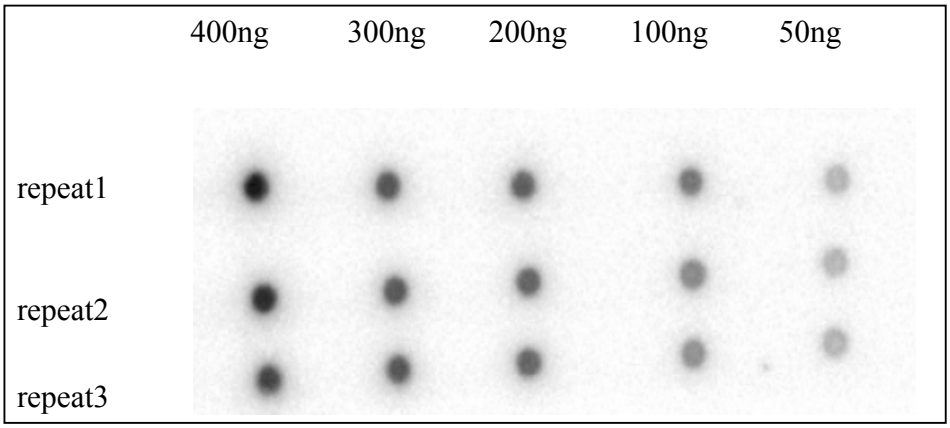


Figure 6. Autoradiogram of different amounts of Chinese DNA vs. Chinese total DNA probe, using American blocking DNA. This is used as a positive control to normalize dot signals.

Hybridization Variation Within and Between Chestnut Generations

A simulation experiment was conducted to test the level of sensitivity of the dot blot technique to genome variation among the chestnut generations. In the simulation experiment, mixtures of Chinese and American total DNAs equal to the average expected ratios for the F1 and 3 backcross generations (1:1 for F1; 1:3 for BC1; 1: 7 for BC2; 1:15 for BC3) were used to simulate average genome content in each generation. On the same blot, an equal amount of genomic DNA pooled from 5 individuals from each generation was applied and probed by Chinese total DNA with blocking DNA from American chestnut (Figure 7). As expected, the American chestnut DNA dot has the least signal intensity, while the Chinese DNA dot has the strongest signal. From F1 to BC3, the intensity of the dots decreased proportionately. When the amount of hybridization to the dots from the simulated DNA admixtures and the bulked DNAs were compared using TotalLab image analysis software, the results showed that the real and simulated mixtures had the same levels of intensity (Table 2), suggesting that on average, the backcross generations have the same ratio of Chinese chestnut genome and American chestnut genome as expected, which the dot-blot technique can faithfully detect.

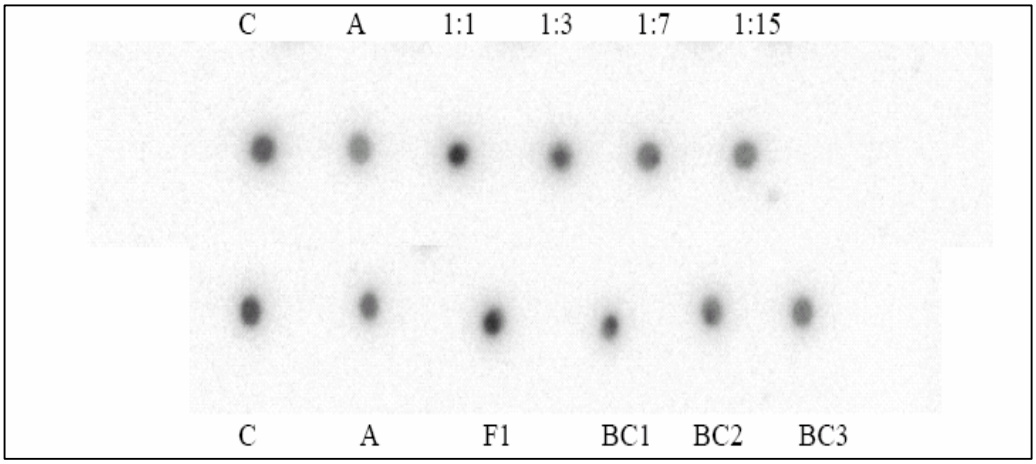


Figure 7. Autoradiogram of Dot Blot hybridization of mixtures of Chinese and American total DNAs vs. Chinese DNA probe, with blocking DNA from American chestnut. First Line: Mixtures of DNAs in the average expected ratios for F1 (1:1) and the 3 backcross generations (1:3 for BC1; 1: 7 for BC2; 1:15 for BC3). Second Line: Bulked DNA samples of six individuals from each generation. Each dot had 100 ng of genomic DNA delivered in 2uL. C, Chinese; A, American; F1, F1 generation; BC1, Backcross1 generation; BC2, Backcross2 generation; BC3, Backcross3 generation.

To determine the extent of variation among individuals within and among backcross generations, a new blot was prepared with DNA dots from 10 individuals from BC1, 12 individuals from BC2 and 12 individuals from BC3, plus the parental DNAs as the internal controls. This blot was probed with labeled Chinese chestnut total DNA, blocked with unlabeled American Chestnut DNA (Figure 8). The signal intensity of each dot for this hybridization was measured in TotalLab (Table 3), and compared to the internal controls. The relative signal intensity, following TotalLab normalization, measured 328 for Chinese DNA and 19 for the American parental DNA. From the distribution of signal intensities among generations (Figure 9), we found, on average, that the BC1 individuals have stronger hybridization than BC2, while BC2 have stronger hybridization than BC3. This trend is as expected from the backcross program, i.e. that in general BC3 individuals have the least Chinese genome DNA remaining. However, much variation in hybridization was detected within each generation, opening the possibility for selection based on DNA content.

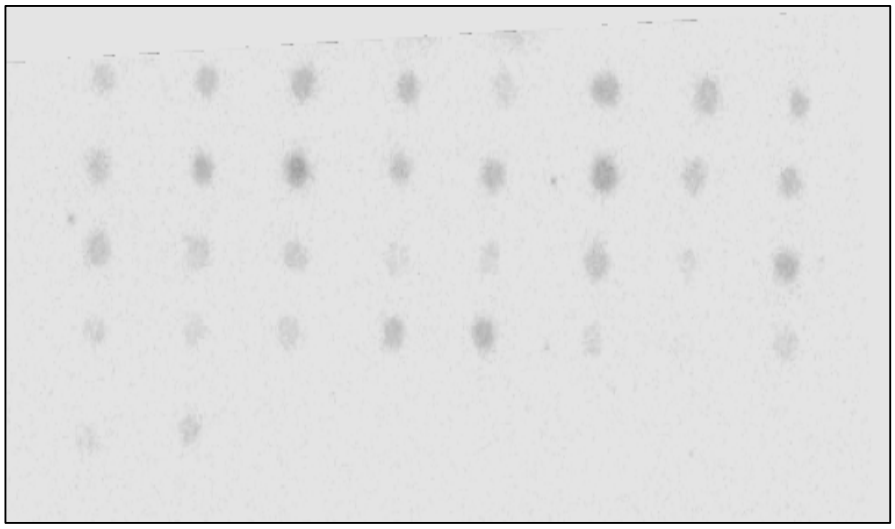
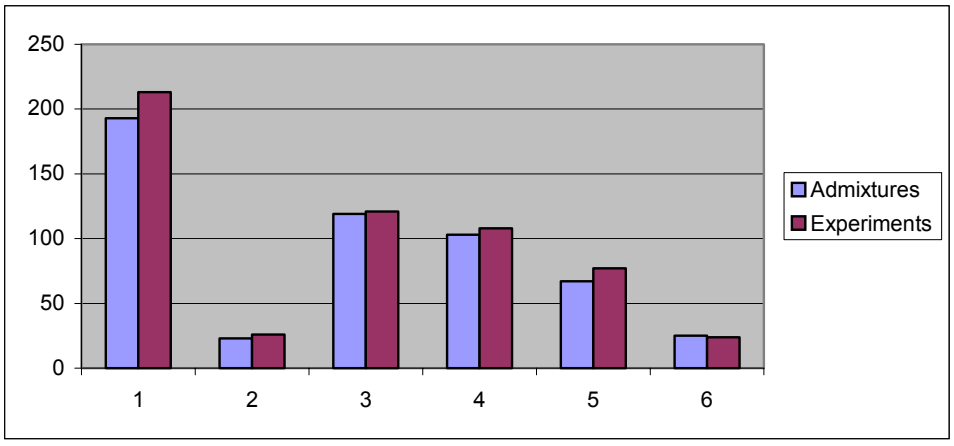


Figure 8. Autoradiogram of Dot Blot individual DNAs from BC1 (10 samples), BC2 (12 samples) and BC3 (12samples) vs. Chinese total DNA probe with blocking DNA from American. Each dot has 50 ng of genomic DNA delivered in 1 uL, all the samples are randomly arranged in this array.

Table 2. Histogram of normalized data for admixtures and experimental samples in Figure 7.



To determine if the differences in hybridization intensities were statistically significant among the backcross generations, we used ANOVA in MINITAB to analyze the measurements by generations. For the result of one-way ANOVA (Figure 10), the P-value was 0.0000, showing that the variation of hybridization among the generations was highly significant. In the dotplot graph of the hybridization measurements (shown in Figure 11), the mean of the BC1 values was significantly greater than BC2, and BC2 was only slightly greater than BC3. For the ANOVA analysis, the variation within each generation was assumed to be equal. The statistical test for equal variation showed that the variation was indeed equal in each generation, although there were two individuals with greater variation than others in BC1, suggesting that there were some experimental errors or random errors in the procedure in those cases. When we increased the sample size (Figure 13), however, the random errors were much smaller than in the experiment with smaller sample size.

Table 3. Values for normalized signal intensity data of hybridization shown in Figure 7 (Control values: 19 for American, 328 for Chinese DNA).

generations / Samples	BC1	BC2	BC3
1	156	94	70
2	121	110	38
3	192	79	33
4	142	104	31
5	148	118	39
6	294	81	32
7	140	119	28
8	286	52	18
9	163	111	57
10	159	65	36
11		68	92
12		79	47

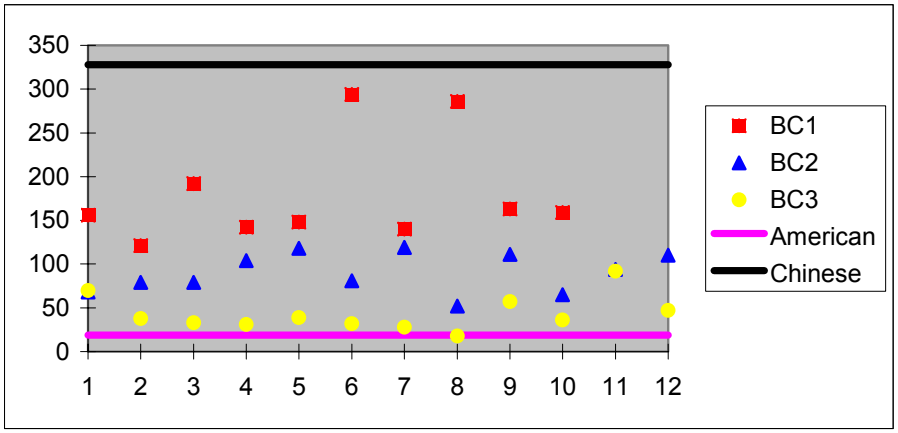


Figure 9. Signal discrimination for dot blot intensities between and within each BC generation, with American and Chinese parent as controls.

One-way ANOVA: nomalizations versus generation

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Analysis of Variance for nomaliza
Source     DF        SS        MS        F        P
generati    2    103780     51890    37.04    0.000
Error      31     43430      1401
Total      33    147210

                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
1          10    180.10     60.76                              (----*----)
2          12     90.00     22.47              (---*---)
3          12     43.42     20.55  (----*---)
                                  ------+---------+---------+---------+
Pooled StDev =    37.43               50       100       150       200
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Figure 10. One-way ANOVA test shows that differences in the signal intensity data between generations are significant.

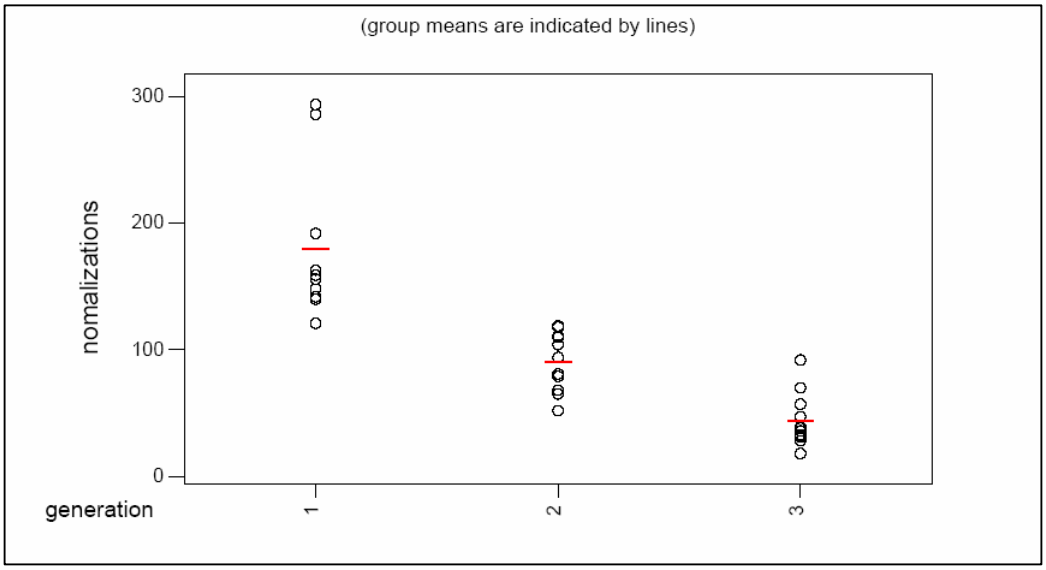

Figure 11. Dotplot of normalized dot blot data by BC generation. Red bars indicate mean values for each BC generation.

Relationship Between Variation in Hybridization Signal Intensities and Morphological Variation Among Backcross Generations

An inherent assumption with use of the dot blot protocol to screen for individuals with greater amount of American chestnut DNA within the backcross generations, was that a strong relationship should exist between variation in DNA at the genome level and the phenotypic, or the morphological, variation within BC generations. In Diskin's thesis (and previous chapter), he measured twenty-four discriminating morphometric characteristics in each of the parental species, the first-generation hybrids, and the three backcross generations. Diskin used principal components analysis was used to develop an Index of Species Identity (ISI) that described the aggregate morphology of the different populations relative to the known American and Chinese chestnut phenotypes. As expected, the morphologies of American and Chinese chestnut were at the extremes measured in his study. The first-generation hybrids were intermediate between the two parental species, and the three backcross

generations had similar morphologies, distinct from Chinese chestnut and largely similar to American chestnut. American chestnut morphology was essentially recovered in the third backcross generation, based on ISI. To determine the relationship between variation in the genomic DNA hybridization data and the morphological variation, we prepared a DNA dot blot with 2 or 3 trees sampled from each bin of the frequencies of the ISI in each BC generation (Figure 12). The hybridization result is shown in Figure 13. All the samples were arranged in random on the blot, with three replications to decrease the hybridization bias and experimental error. The Pearson's correlation coefficient was calculated between the normalized signal intensity measurements and morphological ISI for each individual tree, yielding a value of -0.662, which is statistically significant (Figure 14).

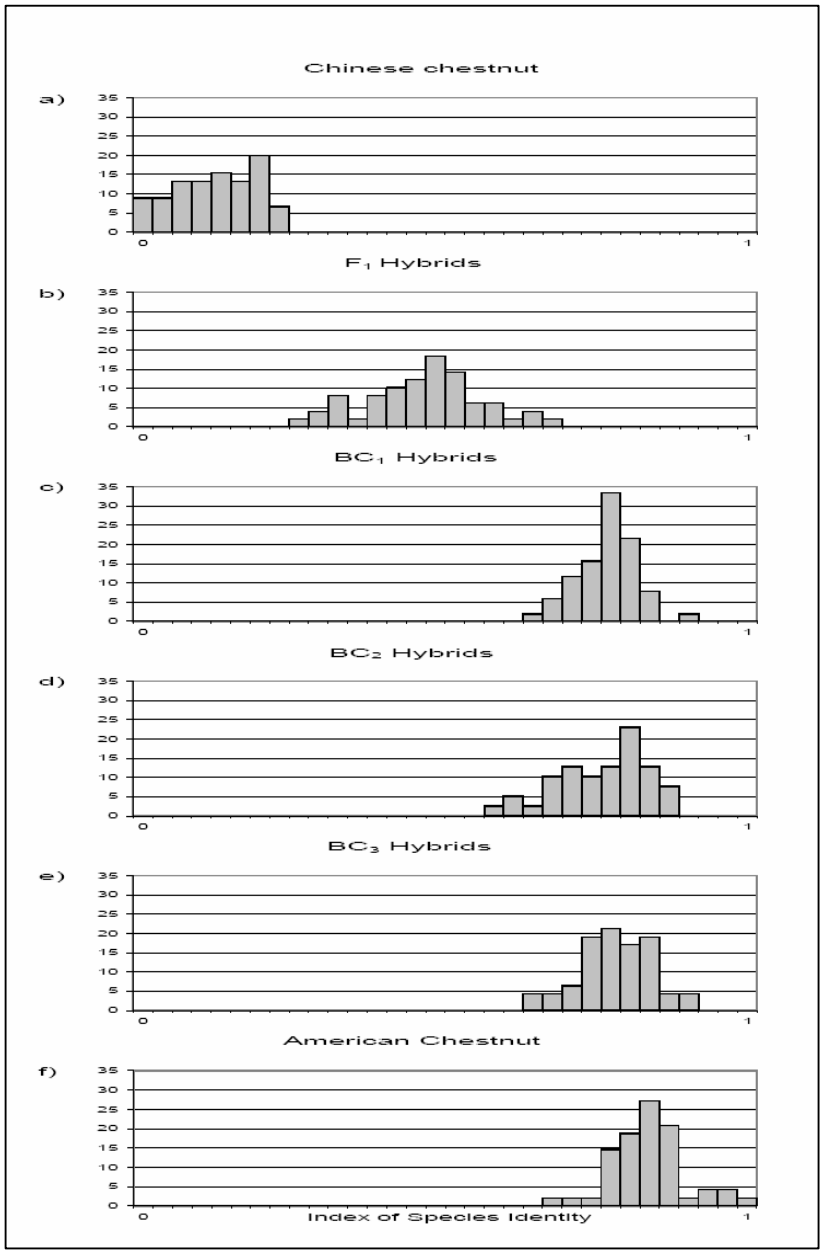

Figure 12. Index of Species Identity. Frequencies of the Index of Species Identity scores are plotted along the y-axis (from Diskin's thesis).

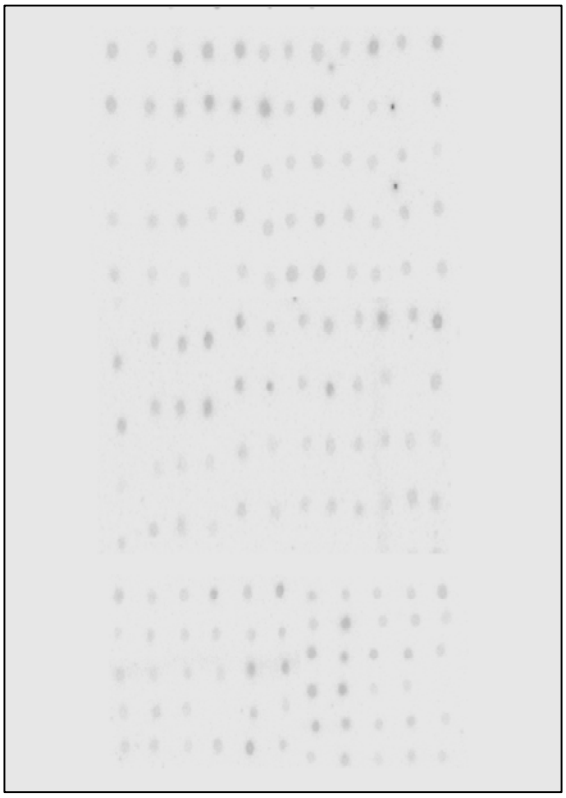

Figure 13. Dot blot of selected samples (82 individuals with 8 controls) from the Diskin morphometric study (Complete random design with two replicates) probed with blocked, total Chinese DNA.

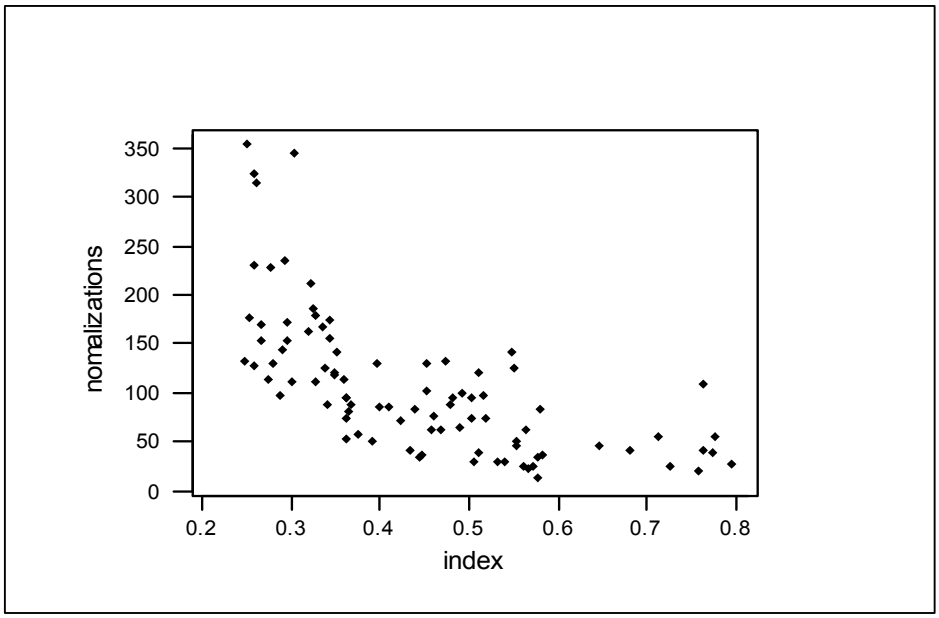

Figure 14. Plot of signal intensity data vs. morphological index data. (Pearson correlation of normalizations and index = -0.662; P-Value = 0.000)

DISCUSSION AND CONCLUSIONS

Genome dot Hybridization Protocol

Genomic hybridization involves extraction of genomic DNA from one of the species of interest, for use as a probe by either Southern hybridization to DNA blots or by *in situ* hybridization to chromosome preparations from the species or hybrids being studied (Orgaard and Heslop-Harrison, 1994). Many of the DNA sequences within the two genomes under investigation may be sufficiently different so that genomic probing discriminates them. Those differences between species may include the different members of classes of repetitive DNA and species-specific DNAs. We do not know the detailed genomic differences between Chinese chestnut and American chestnut, but the results from the hybridization to DNA digests without the blocking DNA showed that their genomes have a high level of similarity, as expected for closely related species. The addition of an excess of unlabelled DNA from the American chestnut parents (blocking DNA) in our experiments substantially increased the specificity of the probe, enabling the two species to be distinguished by hybridization to DNA digests or dot blots. The effect of blocking in our experiments may be due to (a) hybridization between probe DNA (Chinese chestnut DNA) and common sequences in the blocking DNA (American chestnut DNA), (b) hybridization between the blocking DNA (American chestnut DNA) and common sequences on the membrane-immobilized DNA (Dot blot DNA) or (c) a combination of both.

The use of total genomic DNA, in combination with blocking, as a species-specific probe has several advantages. The use of genomic DNA as a probe avoids the need for the time-consuming and uncertain process of screening DNA clones from a library for clones that are specific to the American or Chinese chestnut genomes. Furthermore, it would not have been possible or practical to find enough American or Chinese specific sequences to cover those genomes in the present study. In contrast, the use of genomic probes is simple and straightforward in application, making it practical to develop a screening protocol for application within a large backcross breeding program.

Hybridization Variation Within and Among Backcross Generations

Because the backcrosses were made only to American parents, the Chinese chestnut genome was expected to be progressively diluted as backcrossing progressed. Statistically, it was expected that American genome should comprise on average half of the genome of individuals in the first interspecific hybrid, three-fourths of the genome of the first hybrid generation backcrossed to American, seven-eights of the genome of the first backcross generation backcrossed again to American, and fifteen-sixteenths of the third backcross population, if we assume the parents species have totally different genomes. Also, the variation of genome amounts should become smaller and smaller within each BC generation following successive selections for blight resistance and tree phenotypes. Correspondingly the difference in level of hybridization signal on dot blots should also be observed to decrease in magnitude between generations from the BC1 to BC3 generations ($1/4 \rightarrow 1/8 \rightarrow 1/16$).

Like Diskin's phenotypic ISI index, the DNA dot blot results with American and Chinese chestnut parental species trees in this study were distinct, and represent the two extreme cases, as shown by their scores in Figure 9. This is reasonable, and expected, as the study was based on the known genome differences between American and Chinese chestnut.

The hybridization signals of the populations measured in this study were summarized by their normalized data (Table 3). The progression towards American-like genome in each successive hybrid generation from BC1 to BC3 was apparent from the decrease in the means of the normalized signals (Figure 11). Also, as expected, the decrease in mean values from BC1 to BC2 was much greater than the decrease in values from BC2 to BC3. The decrease in magnitude of change towards the American chestnut genome value among backcross generations fits expectations: each successive backcross generation is on average more American than the previous generations and the genome of the third

backcross generation (mean = 43.42) approaches most closely that of American chestnut (normalization=19).

The ANOVA results showed that the mean differences among the BC generations were significant. This proved that the variation in dot blot hybridization is related to genomic variation, and not caused by experimental errors or random errors. The ANOVA test result of equal variance among each BC generation is not what we expected based on statistical considerations, however. The reasons for the equal variance in genome content among generations may be (1) that the sample size was not big enough to represent the whole population, bringing bias into the population sampling; or that (2) the genome differences between Chinese and American chestnut are actually too small to reliably distinguish the variances among the generations at the scale of dot blot sensitivity or that (3) additional variation is produced at each generation by recombination events during gamete formation.

Relationship Between the Hybridization Data and Morphology Data

To be able to screen for the individuals which are more American- like in the BC generations based on the results of dot blots, one should show that the variation in DNA content within generations is strongly related to the morphological variation. The ideal result would be a one to one relationship (Pearson's correlation coefficient=1 or -1).

In the project conducted by Matthew Diskin (Diskin 2003), an Index of Species Identity (ISI) was used to describe the aggregate morphology of the different populations. In our study prepared dot blots from samples selected from among those used by Diskin. The relationship between the dot blot hybridization data that we obtained and Diskin's ISI values was strong (Pearson's correlation coefficient = -0.662). A possible reason for this strong correlation could be that most of the genome sequences detected by the dot blot technique are expressed coding sequences that evolved at the same rate or along with the evolution of the morphological differences between the species. The negative value of the relationship is logical, because the ISI is positively related to American characteristics, while the hybridization data is negatively related to the amount of American genome DNA.

The relationship between morphological index and genomic dot blot signal intensities was not one to one, however, indicating that it is not possible to predict the morphometric differences between trees with 100% success based just on the differences in dot blot signals. Two possibilities could account for this. The first possible explanation arises from the fact that not all of the morphological variation that represents the species-specific characters were used to generate the ISI. If the morphological characteristics measured were not comprehensive enough, this could cause a bias in the ISI analysis. The genome-level variation assessed by dot blots should, in theory, be able to uncover differences in many more characteristics than is possible though phenotypic evaluation.

A second possible explanation for the differences between the ISI and dot blot results could be that the genomes of Chinese chestnut and American chestnut are highly similar because they are closely related. DNA sequences in the Chinese chestnut genomic probe that are highly similar to American chestnut sequences will be removed during the blocking step even though they may have very different expression patterns and cause different morphological characteristics. To minimize this concern, high stringencies were used in the dot blot filter washing steps and in probe blocking to ensure that only virtually identical sequences between American and Chinese chestnut species would be removed from the genomic probe.

In summary, this project has demonstrated that the dot blot technique can produce similar results to that obtained by the more painstaking and lengthy assessment of genotypes based on assessment of morphology for individuals in American chestnut backcross generations. The convenience, sensitivity and rapidity of the dot blot approach should make the technique more suitable than phenotyping for screening large populations of trees and seedlings for American vs. Chinese genetic makeup. The observation that a significant amount of variation in dot blot signal intensity was observed among individuals in all three of backcross generations, indicates that the dot blot technique would be useful

for selecting individuals with the greatest amount of American genome at each generation. The dot blot tool could thus greatly accelerate the goal of breeding blight resistant trees that have regained the genetic makeup of the American chestnut species.

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