Some Resistance Genes Against *Cryphonectria parasitica* May Be Strain-Specific

Timothy S. McKechnie

Abstract: The goal of The American Chestnut Foundation (TACF) is to produce hybrid American-type chestnuts (*Castanea dentata*) with adequate, long-lasting resistance to *Cryphonectria parasitica,* the fungus that causes chestnut canker disease. TACF is using two variations of a single backcross breeding scheme, which is designed to transfer resistance from Chinese chestnut (*Castanea mollissima*). Achievement of adequate, long-lasting resistance under either variation of the breeding scheme may depend on having knowledge of interactions between Chinese resistance genes and different strains of the pathogen. Third backcross hybrids derived from the "Clapper" source of resistance, along with Chinese, American, and F1 (Chinese x American) hybrid control trees were inoculated with mycelium of two fungal strains. Resulting cankers were measured at 11 weeks. Two independent lines of analysis suggest the existence of one or more strain-specific resistance genes. First, strain-specific phenograms show distinct inflection points and randomization of the second strain. Second, individual trees with extreme forms of strain-specific resistance phenotypes are more numerous than predicted by models lacking strain-specific genes. The existence of strain-specific genes can't be proven with the present data set because it was not an experiment designed to reveal strain-specific effects. However, if strain-specific genes are confirmed in studies of long-term resistance of TACF hybrids, there will be implications for the purpose and design of the central TACF breeding program in Meadowview, VA, the purpose and design of the regional breeding programs in eight states, and whether more than one breeding scheme may be advisable on the central and regional levels.

Keywords: Resistance, interactions, *Cryphonectria parasitica*, *Castanea dentata*, *Castanea mollissima*

INTRODUCTION

The goal of The American Chestnut Foundation (TACF) is to produce hybrid chestnut trees with many of the qualities of American chestnut (*Castanea dentata*) and also adequate, long-lasting resistance to *Cryphonectria parasitica,* the fungus that causes chestnut canker disease. TACF is using two variations of a single breeding scheme (Hebard 2005) one of which involves passing resistance from Chinese chestnut (*Castanea mollissima*) through a single BC1 hybrid followed by two or more backcrosses to American chestnut followed in turn by an intercross intended to create homozygous resistant hybrids that are expected to breed true for high resistance. The second variation passes resistance from a single Chinese chestnut through twenty F1 trees and is otherwise the same as the first variation. Evaluation of the resistance being developed under either variation of the scheme so far has not including testing for interactions between resistance genes and different strains of the pathogen.

Three studies potentially capable of detecting interactions between isolates of *C. parasitica* and individual Chinese chestnuts (*Castanea mollissima*) have been published. A phenotype-level study of aggressiveness of two *C. parasitica* isolates on Chinese chestnut (in China) reported that the two pathogen isolates had statistically identical aggressiveness and no isolate-by-tree interactions (Ling, Xiahong et al. 2002)[1]. However, it's not clear whether the detached-stem assay used was reliable[2]. In contrast to the study mentioned above, there are two published reports that support the existence of phenotype-level isolate-by-tree interaction between *C. parasitica* isolates and Chinese cultivars. One study found significant isolate-by-cultivar interactions at the seedling stage[3] (Huang, Carey et al. 1996) using three isolates and a whole-tree assay. The other report used whole trees at least 25 years old (Anagnostakis 1992) and two isolates but did not explicitly test whether the interactions observed were statistically significant. Turning attention to American chestnut, a genetic-level test on whole trees (saplings) indicated no isolate-by-tree-family interactions among half-sib Americans (Huang, Carey et al. 1996).

Remarkably, until the present report, there has been no published study of phenotype-level or genetic-level isolate-by-tree interactions on American chestnut hybrids. The lack of attention to strain-by-hybrid interactions is remarkable because interactions are more likely to be observed in hybrids than in pure species and because the existence of interactions is a significant cause for concern in a breeding program (Nelson 1978).

MATERIALS AND METHODS

TACF uses two strains of *C. parasitica* to test trees for resistance. Inoculation with the strain named "Ep 155" (hereafter "Ep") usually results in larger cankers than inoculations with the other strain, named "SG1 2-3", (hereafter "SG").[4]

The canker measurement data in this article are from a set of third backcross (BC3) progeny derived from "Clapper", which is a BC1 (Chinese X American) X American and is one of TACF's main sources of resistance. The BC2 parent is identified by TACF as "CL287". The Pennsylvania American[5] parent of the BC3s is known as "Ort". All trees were planted in the spring of 1997. They were grown[6] and inoculations performed according to standard TACF

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[1] Thanks to Bruce Levine of the Maryland Chapter and to Susan You, Duke University, 2005 summer intern with the PA Chapter of TACF, for the detailed translation.

[2] This report was based on mycelial inoculation of detached Chinese chestnut stem segments kept under (probably) anaerobic conditions in plastic bags, which were stored in an incubator at 25°C and 70% humidity. Not surprisingly, a fermented odor was noted for the larger cankers. There was no effort to demonstrate that the assay reflected resistance on whole trees.

[3] Huang *et. al.* used three isolates, fifteen seedlings per cultivar, one inoculation per seedling, and performed ANOVA on measurements taken at six weeks, which is after healing had begun, and the cankers were shrinking.

[4] "Ep 155" is American Type Culture Collection isolate number 38755 (http://www.atcc.org/). "SG1 2-3" was isolated near Meadowview, VA. It was chosen for testing purposes because it was one of the least aggressive isolates when inoculated on American chestnut.

[5] Possibly a European / American hybrid based on leaf characters. This is a point of controversy.

[6] The trees were grown by Ann and Dr. Robert Leffel near Brogue, PA.

procedures for backcross progeny[7], which involves two inoculations of each strain on every tree. All trees were inoculated on May 27, 2000 and measured between August 14 and 20, 2000, when the inoculations were 11-12 weeks old. Canker sizes reported in this article are the average of width and length measured without scraping the bark.[8]

Out of 149 BC3s originally planted, only 84 survived long enough to be inoculated. Only 76 of these provided two measurements for both SG and Ep and were included in the analysis. This was the first BC3 orchard at this location, and natural *C. parasitica* disease pressure appeared to be low (personal observation).

Controls trees were planted as same-age seedlings in a randomized fashion among the BC3s and were inoculated and measured at the same time as the BC3s. The eight Chinese chestnut control trees were half siblings, produced by open pollination on "Chinese D89" near Meadowview, VA. The American chestnut controls trees were open pollinated from various Pennsylvania trees. Three F1 controls were derived by controlled pollination of uncharacterized Chinese "Leffel North" x American "Ort". Five F1 control trees were derived by controlled pollination of Chinese "Meiling" KY175 x American TPE17.

RESULTS AND DISCUSSION

The existence of strain-specific resistance genes can't be proven with the present data set. The present data are based on canker measurements of a set of BC3 trees that were part of the TACF breeding program, not an experiment designed to reveal strain-specific resistance genes. The purpose of this paper is to argue that further testing is warranted.

Another important point is that the cankers discussed in this paper were only 11-12 weeks old when measured. Short-term observations of canker size do not always predict long-term resistance (Grente 1961). However, preliminary results (not shown) based on measurements of one year old cankers on a second Clapper BC3 family show patterns similar to those summarized by this paper.

With the above caveats in mind, phenograms are a good starting point for analysis because they provide a visual data summary which can often be used to formulate easily testable hypotheses.

When all four canker measurements, two for SG and two for Ep, are averaged for each BC3 tree, the resulting SG+Ep phenogram, Chart A, shows relatively little evidence for discrete gene effects. There are no dramatic inflection points or plateaus. The feature most suggestive of the effects of discrete genes is at the intersection of a small BC3 phenotype plateau with the upper limit of F1 control tree SG+Ep averages at 7.1cm, corresponding nearly exactly to a progeny ratio suggesting three unlinked equivalent[9] genes.

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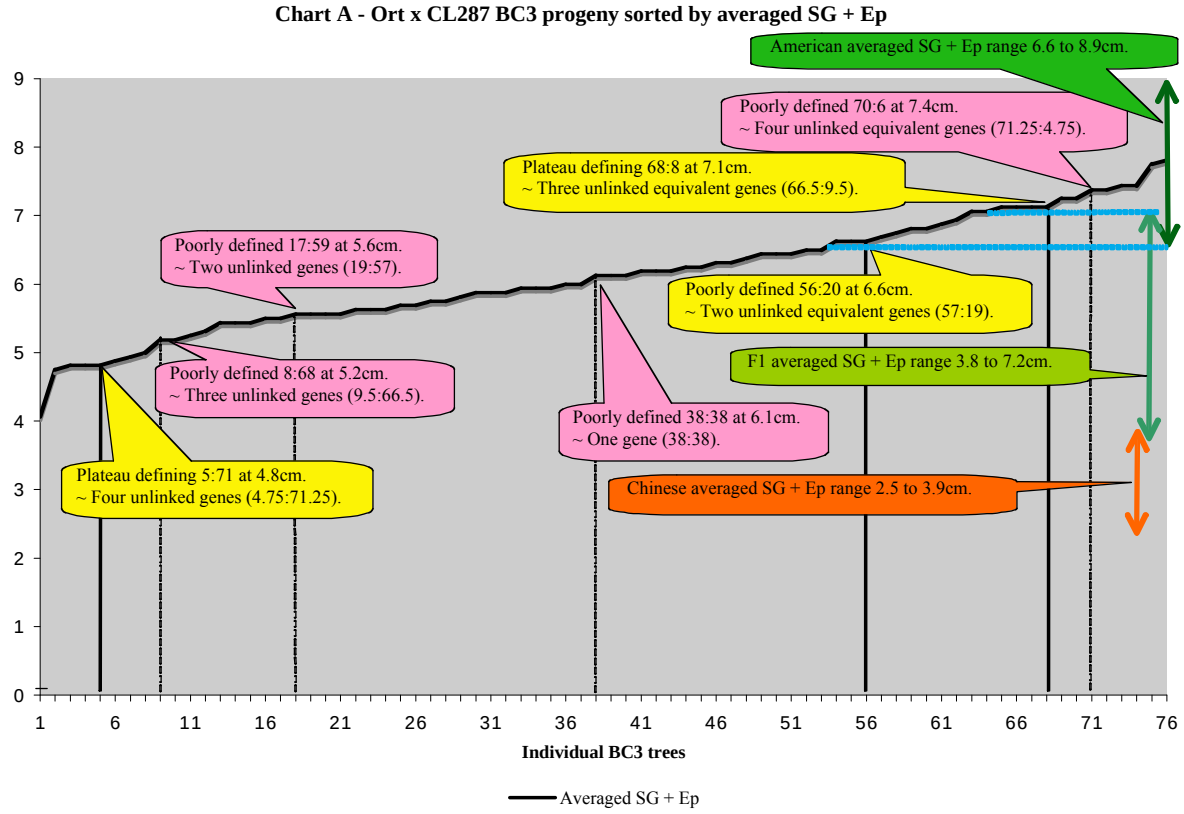
[7] See written procedures at http://chestnut.cas.psu.edu/Breeding.html.

[8] Thanks to Sara Fitzsimmons and Ann Leffel for their careful measurements.

[9] "Equivalent" here means that any one of the three hypothetical genes, acting alone, is capable of providing at least this level of resistance. Note that all three genes could be strain-specific.

When the two SG measurements are averaged for each BC3 tree, the resulting phenogram, Chart B, has at least four features that can be interpreted as the effects of unlinked genes acting alone. The largest plateau, consisting of eight trees with SG averages exactly at 4.5cm, ends nearly exactly where an inflection point defining a 1:1 ratio would be expected. The second largest plateau, consisting of five trees with SG averages exactly at 5.1cm, ends nearly exactly where an inflection point defining the effects of two unlinked equivalent[5] genes would be expected. Two inflection points are observed near the intersection of the phenogram with limits defined by the upper SG range of F1 control trees and the lower SG range of American control trees, nearly exactly at progeny ratios defining three and four unlinked equivalent genes respectively.



Assuming sources of non-genetic variation and gene x environment interaction are relatively small, if Ep canker size is governed by the same genes as the response to SG, one might expect the Ep phenogram to show similar features as the SG phenogram. However, Ep response is seen to fluctuate in an apparently random fashion between Chinese level resistance and American-level resistance across the entire range of SG response (dashed line, Chart B). These dramatic fluctuations in Ep phenotype, to the extent they are genetic, suggest assortment of at least one Ep-specific gene with large effect that is unlinked to any of the putative genes acting on SG.

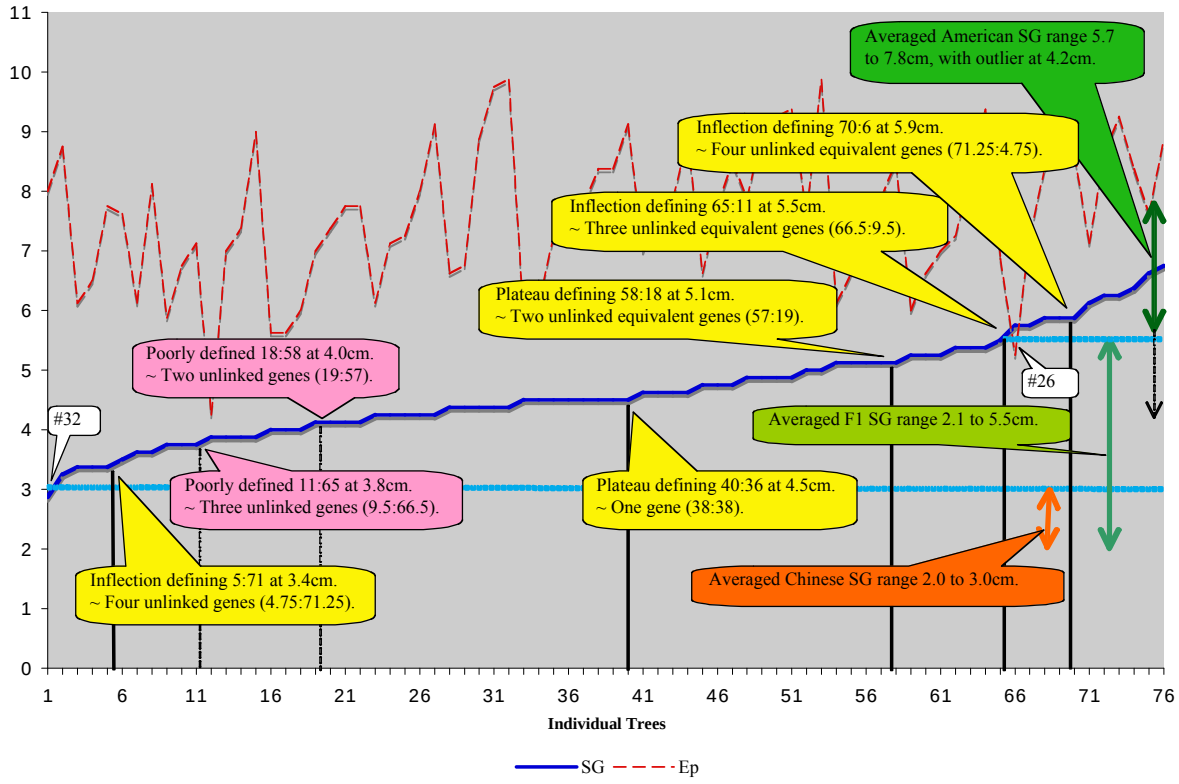
Limiting attention to BC3 trees ranked 19 to 38 in Chart B, the variation in their SG averages is easy to explain in terms of measurement error, Chart C.[10] In contrast, the variation of their Ep

[10] The standard deviation of all BC3 measurements (within strain, within tree) from their average is 0.44cm. The standard deviation of the average is $0.44/2^{1/2} = 0.31$. The deviation from the mean within this region is smaller: 0.22cm.

averages is too large to explain in terms of measurement error. The same observations apply to trees ranked 39 to 57 in Chart D. Whatever is causing the variation in Ep canker size in Charts C and D is affecting the whole tree, or at least a ~1.2 meter long section of the trunk.

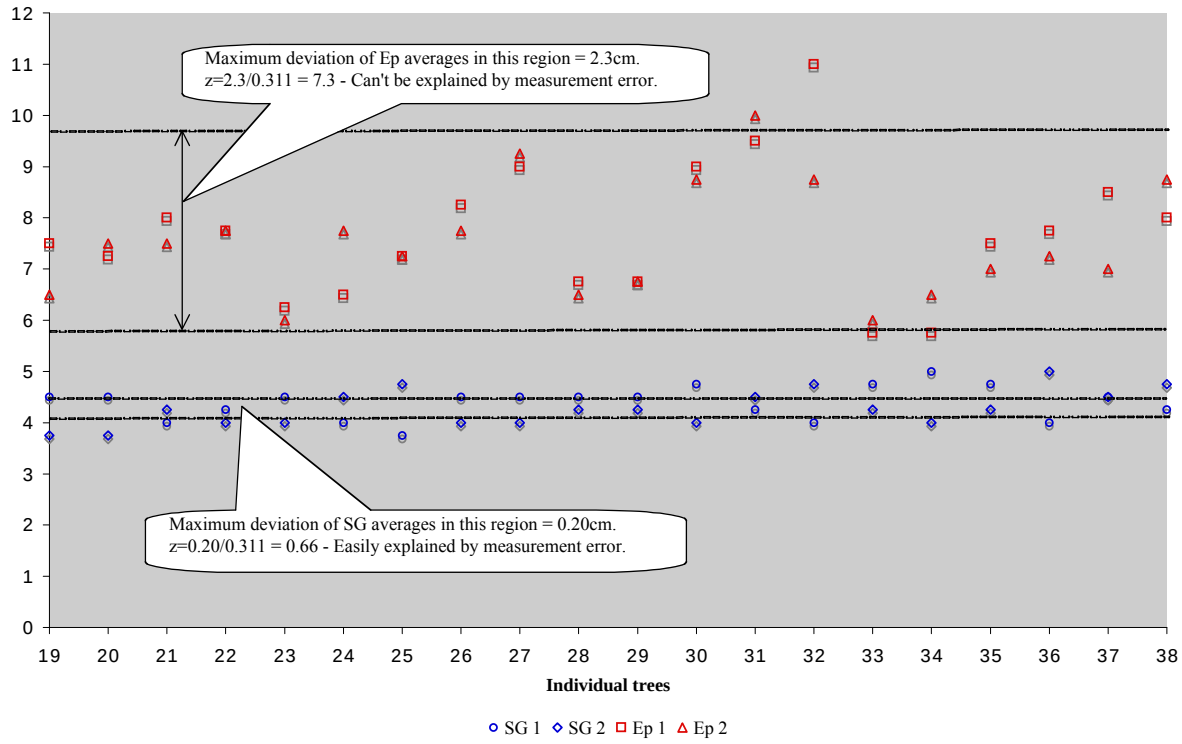
Because of their position in the SG phenogram and their low SG variation, it's reasonable to hypothesize that the sets of progeny considered in charts C and D might have the same genotype with respect to SG resistance. Because of the high variation in Ep canker size between trees and the reproducible behavior of Ep cankers within each tree, it's also reasonable to suppose that Charts C and D show the effects of one or more randomly assorting, Ep-specific resistance alleles.

Chart B - Ort x CL287 BC3 sorted by averaged SG



The Ep phenogram, Chart E, also shows features that can be interpreted as the effects of discrete genes. The largest plateau, consisting of six trees with Ep averages exactly 7.6cm, ends nearly exactly where an inflection point defining a 1:1 ratio would be expected. A plateau consisting of five trees with Ep averages exactly 8.4 cm ends nearly exactly where an inflection point defining a 3:1 ratio would be expected. The upper limit of F1 Ep response (8.9cm) intersects the BC3 phenogram nearly exactly where a 7:1 ratio would be expected. A dramatic inflection point adjacent to the plateau at 8.4cm suggests the action of a pair of linked resistance genes with large effect. Another dramatic inflection point adjacent to a plateau at 6.1cm suggests the action of three additive genes. The SG response fluctuates in an apparently random fashion between Chinese level resistance and American-level resistance across the entire range of Ep response (dashed line, Chart B). If those SG fluctuations have a genetic cause, they suggest at least one SG-specific gene that is unlinked to any of the putative genes acting on Ep.

Chart C - First subset of BC3 canker sizes, sorted by SG average.



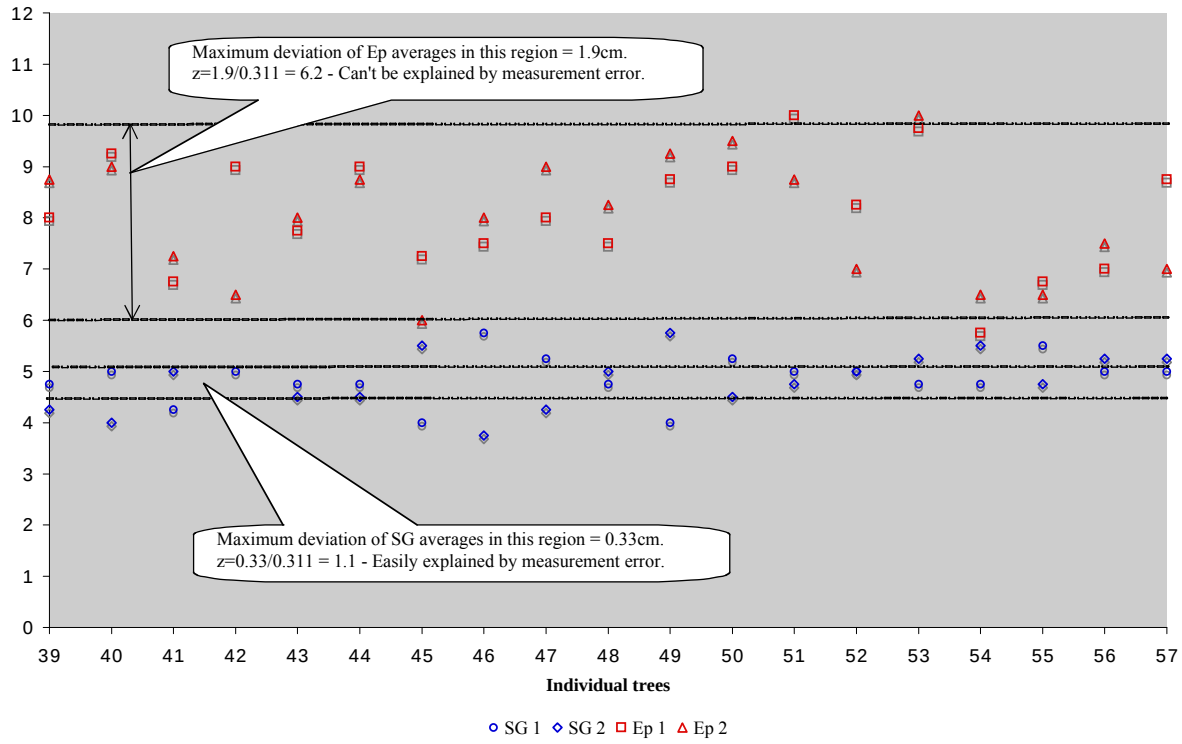
Compared to the SG+Ep phenogram, both the Ep and SG phenograms have more features suggestive of the action of discrete genes and these features are more dramatic. One interpretation might be that the SG+Ep average (over four measurements) removes the random effects present in the SG and Ep averages (over two measurements). However, as noted above, duplicate cankers for SG and Ep are highly reproducible. An alternative interpretation is that examining response to individual strains separately may clarify gene action, which in turn suggests that the resistance genes of largest effect may be strain-specific.

SG and Ep canker sizes in the BC3 are correlated (Pearson correlation of 0.84). That correlation could be interpreted in terms of one or more resistance genes effective against both SG and Ep. Alternatively or in addition, the correlation could be caused by environmental effects and/or pairs of strain-specific genes that are on the same chromosome, i.e., linked pairs consisting of one SG-specific resistance gene linked to one Ep-specific resistance gene.

Based on analyses like these, future crosses will be performed with the intent to isolate single strain-specific alleles.

A variety of other genetic modeling approaches also suggest the existence of strain-specific resistance genes in this cross. For example, it's possible to compare the observed number of trees with strain-specific resistance phenotypes with the number predicted under the assumption of random non-genetic phenotype fluctuations.

Chart D - Second subset of BC3 canker sizes, sorted by SG average.



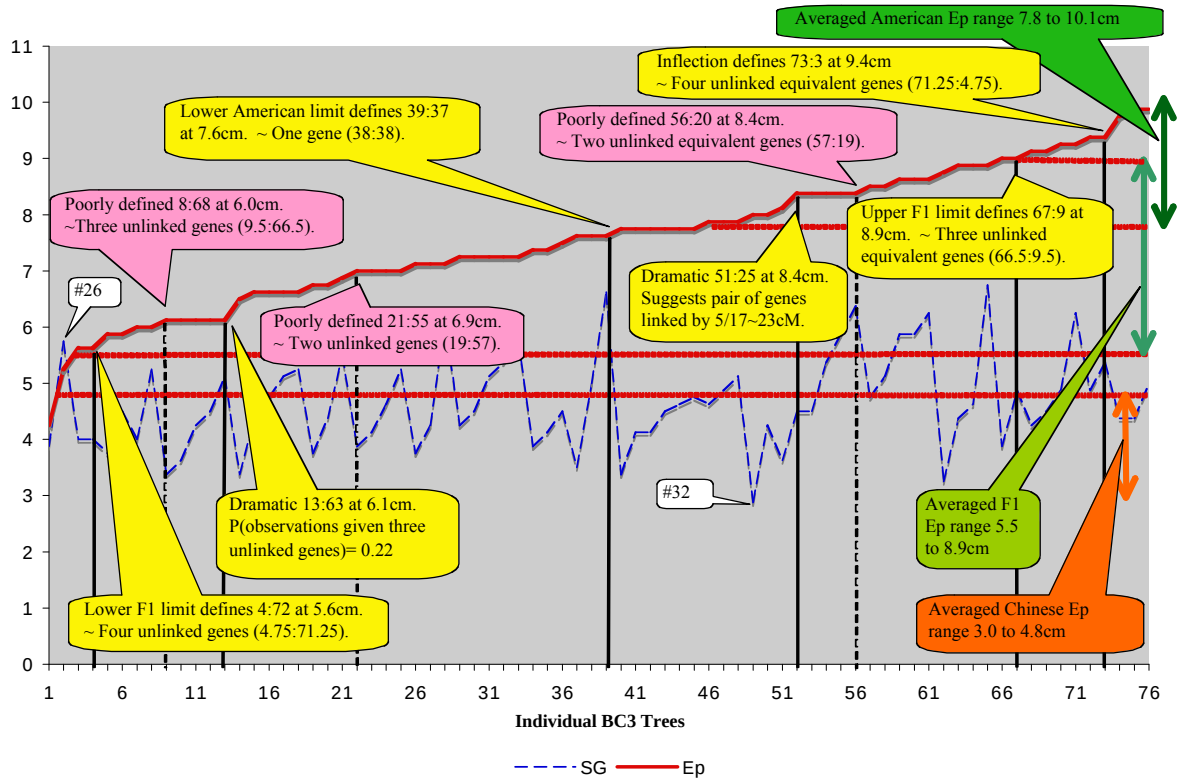
Suppose resistance is defined as any tree with cankers significantly smaller than the American controls.[11] In that case, ten out of the 76 BC3s could be considered to exhibit SG-specific resistance phenotypes[12] and seventeen to exhibit Ep-specific resistance phenotypes[13]. Prediction of the expected number of strain-specific phenotypes caused by non-genetic fluctuations involves three steps: (1) Assume a genetic model, preferably one that fits the over-all BC3 data. (2) Estimate the frequency of non-genetic strain-specific phenotypes for each genotype in the model using control tree data and the bivariate normal distribution. (3) Multiply the above frequency by the associated normalized Chi-square probability and integrate over all possible outcomes.

According to the modeling process described above, no simple genetic model based on non-strain-specific genes can fully explain the observed number of strain-specific phenotypes, Table A. The only simple model (considered here) providing a reasonable fit to the overall SG+Ep averages (two equivalent genes) predicts one-third as many Ep-specific phenotypes as were observed. An over-all numerical estimate of certainty for the above conclusion may be possible

[11] For z=1.65 at the 0.05 confidence level, SG<4.32cm and Ep<7.59cm. Under this definition, resistant trees will have phenotypes at the F1 or better level.
[12] That is, SG average less than 4.32cm and Ep average greater than 7.59cm.
[13] That is, SG average greater than 4.32cm and Ep average smaller than 7.59cm.

but is irrelevant. That's because the main source of uncertainty in this process lies in the assumptions made while constructing the models.[14]

Chart E - Ort x CL287 BC3 sorted by averaged Ep



The modeling process described above can be repeated based on a more extreme form of strain-specificity: Chinese-level resistance for one strain and American-level resistance for the other strain. Again, no simple genetic model based on non-strain-specific genes can explain the observed cases strain-specific phenotypes, Table B. In particular, all of the simple models (considered here) predict a very low probability (<0.008) for the Ep-specific phenotype that was observed in one BC3 tree (orchard #26), Charts B and E.

Note that mistakes at inoculation time, such as putting Ep in all four inoculation holes could explain the observations on tree #26 if it had Chinese-level resistance against Ep. However, the inoculation procedure is well-designed to avoid that kind of error. One person inoculates the SG strain and another person inoculates Ep: both people would have to miss such a mistake.

[14] The principle assumptions in Tables A and B are that SG/Ep variation and correlation within groups of control trees are not caused by variation in resistance genes and that these parameters apply to BC3 genotypes. "Lack of genetic variation within groups of control trees" may very well be a false assumption. However, that's not a problem for the over-all argument since the resulting low SG/Ep correlations would only serve to increase the estimate of non-genetic error rate.

Table A - Expected numbers of strain-specific phenotypes defined as: "Cankers of one strain the same as on American controls and cankers of the other strain significantly smaller than American controls, (p=0.05, z>1.65)".

	Predicted frequency among 76 BC3 progeny:		Overall model fit[b]
Models[a]	SG<=5.5cm, Ep>=9.0cm	SG>=5.7cm, Ep<=8.9cm	
Three equivalent genes predict: 66.5:9.5	9.0	5.7	0
Two equivalent genes predict: 57:19	8.3	5.4	0.29
One gene predicts: 38:38	6.6	4.7	0.0005
Two additive genes predict: 19:57	4.9	4.0	0
Three additive genes predict: 9.5:66.5	4.1	3.6	0
Actual cases of specificity observed:	10	17	---

a - Models assume that each resistance allele provides full (for equivalent genes) or partial (for additive genes) F1-level resistance.
b - Chi square fit to the 53:23 observed for averaged SG+Ep cankers significantly smaller than American.

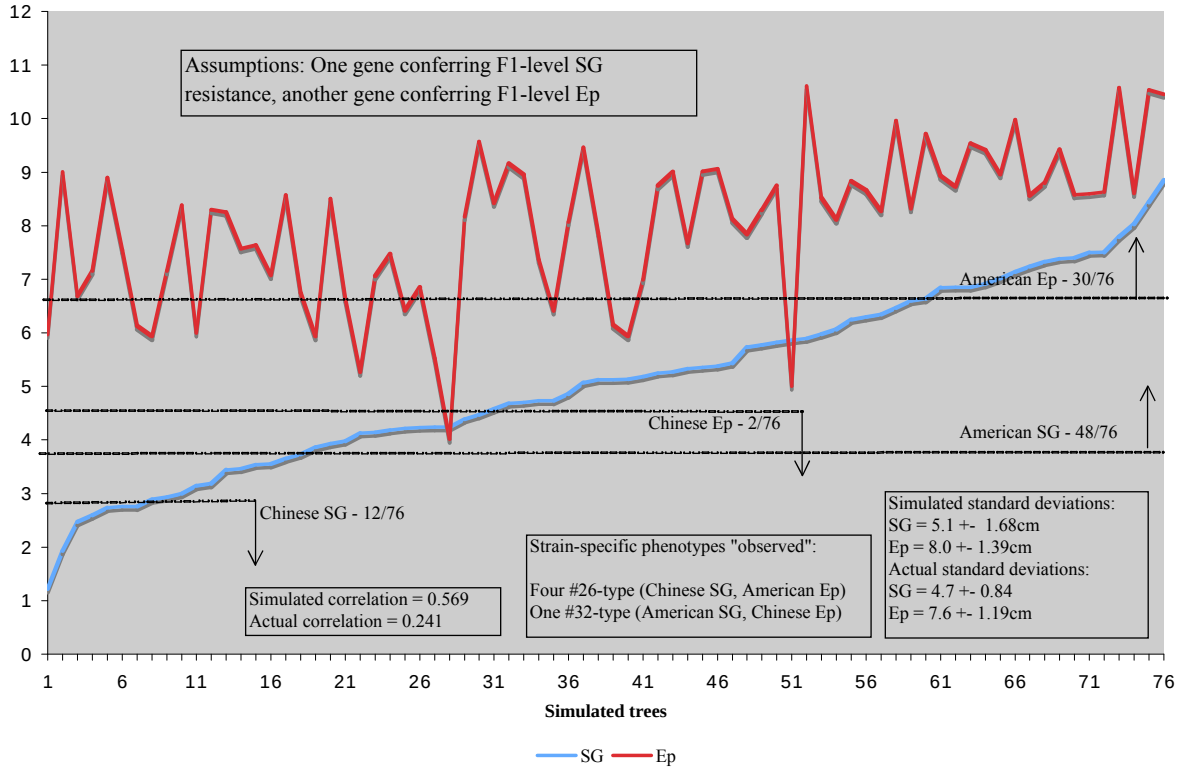
Table B - Expected numbers of strain-specific phenotypes defined as: "Cankers of one strain the same as Chinese controls and cankers of the other stain the same as American controls, (p=0.05, z>1.65)".

	Predicted frequency among 76 BC3 progeny:		Overall model fit[c]
Models[a]	SG<=3.23cm, Ep>=7.59cm	SG>=4.32cm, Ep<=5.17cm	
One gene predicts: 38:38	<0.19	<0.0076	0
Two additive genes predict: 19:57	<0.28	<0.0076	0
Three additive genes predict: 9.5:66.5	<0.33	<0.0076	0.0009
Four additive genes predict: 4.75:71.25	<0.35	<0.0076	0.024
Five additive genes predict: 2.4:73.6	<0.36	<0.0076	0.12
Six additive genes predict: 1.2:74.8	<0.37	<0.0076	0.27
Actual cases of specificity observed:	1 (#32)	1 (#26)	---

c - Chi square fit to the 0:76 observed for averaged SG+Ep smaller or equal to largest Chinese canker.

Data simulations such as that shown in Chart F suggest that just one Ep-specific gene and one SG-specific gene are sufficient to explain the observed cases of extreme strain-specific phenotypes noted in Table B. Over the course of ten simulations, an average of five cases of Chinese-level strain specificity like BC3 orchard #32 and 1.6 cases like orchard #26 were generated. In contrast, simulations without strain-specific resistance genes failed to produce any cases like orchard #26. Even adjustments to the SG/Ep correlations and the standard deviation of SG separately from Ep did not produce a strain-specific phenotype like orchard #26 or an otherwise reasonable model of observed data.

Chart F - Synthetic Ort x Clapper 287 data, sorted by SG.



The several methods of analysis used in this paper all suggest the existence of resistance genes with a moderate or high degree of strain-specificity. Phenogram analysis suggests that such strain-specific genes may have large effects on phenotype when acting alone. These are properties of R genes. Properties suggestive of R genes were also recently discovered in a canker disease of eucalyptus caused by *Cryphonectria cubensis* (van Heerden, Amerson et al. 2005).

Although never explicitly stated in print, the TACF breeding program has been based on the assumption that resistance in crosses between Chinese and American chestnut is not strain specific. It's possible to find broad claims in the literature that resistance to necrotrophic pathogens like *C. parasitica* is usually not strain specific, but such claims are rare. One such claim (Glazebrook 2005) is stated without explanation or reference. Another (Brasier 1987) presents an argument based on species-level resistance, which isn't a genetic test of strain-specificity. Resistance genes thought to be non-strain-specific do exist for necrotrophs, but such genes are found to be present with genes that are strain-specific (Newton and Crute 1989). Mechanisms for strain-specific susceptibility to necrotrophs have been studied on the molecular level (Thomma 2003; Ito, Tanaka et al. 2004). Resistance genes against specific strains of a necrotroph have been mapped (Cho and Muehlbauer 2004).

A literature survey of hardwood canker pathology suggests that belief in lack of specificity, to the extent such a belief exists, is probably based on long-term survival of wild genetic material, often clonally-propagated. Wild trees, because they are long-lived, can reasonably be expected

to harbor a diverse set of resistance genes against a wide spectrum of necrotroph strains adapted to that host. Therefore, long-term survival of such genetic material is not good evidence that resistance genes lack strain-specificity. A proper test for strain specificity involves crosses between resistant and non-resistant trees followed by tests with individual strains, as is being done in the TACF breeding program.

If strain-specific genes are confirmed in studies of long-term resistance of TACF hybrids, there will be implications for the purpose and design of the central TACF breeding program in Meadowview, VA, the purpose and design of the regional breeding programs in eight states, and whether more than one breeding scheme may be advisable on the central and regional levels. To the extent that TACF's present breeding method is based on incorrect assumptions, additional breeding methods should be considered which do not make such assumptions (Borlaug 2000; Leffel 2004). Apparently only silvicultural tests are planned before large-scale deployment of hybrids (Steiner, Ellingboe et al. 2004). Such silvicultural tests (no inoculations are called for) might or might not expose TACF hybrids to a wide variety of strains. The large-scale deployment itself is seen as the best way to test durability of resistance (Hebard 2004). If long-term resistance is strain-specific, this idea should be reconsidered.

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