

## Genetic and Genomic Resources for Mapping Resistance to *Phytophthora cinnamomi* in Chestnut

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### Abstract

Root rot (caused by *Phytophthora cinnamomi*) and chestnut blight (caused by *Cryphonectria parasitica*) are the two most destructive diseases affecting American chestnut, *Castanea dentata*. Therefore, breeding for resistance to both pathogens simultaneously is essential before the American chestnut can be restored to its full native range. Using combined genetic and genomic approaches, resistance to *C. parasitica* (*Cp*) has been mapped to three quantitative trait loci (QTLs) in chestnut. In addition, a marker set covering the chestnut genome has been generated for implementation in breeding for *Cp* resistance. Although *P. cinnamomi* was introduced to the USA far earlier than *C. parasitica*, an effort to breed for resistance to this pathogen has been initiated only recently. Selection of parental genotypes with a high inter-generational transmission rate of resistance to *P. cinnamomi* (*Pc*) allowed initiation of genetic studies. In pilot experiments with a limited number of progeny derived from AdairKY1 × GL158, a QTL for resistance (source Chinese chestnut, *C. mollissima* ‘Nanking’) to *Pc* was mapped to linkage group E (LG\_E). Subsequently, three backcross families (HB1, HB2, and MK5) were selected from another source of resistance (*C. mollissima* ‘Mahogany’) for map construction and association analysis with 22 markers from LG\_E. Our preliminary analyses confirmed the presence of a *Pc* resistance QTL on LG\_E. In 2012, extended mapping populations (up to 200 individuals) representing the Mahogany and Nanking lineages of resistance were planted for phenotyping of *Pc* resistance and QTL mapping. Together, these materials and analyses should help resolve the location of

## the QTLs for resistance to *Pc* and test their co-location between two important sources of resistance in chestnut.

### INTRODUCTION

At one time, the American chestnut, *Castanea dentata*, was the dominant tree species throughout the forests of the eastern United States (Russell, 1987). It was a significant contributor to local economies valued both as a lumber tree and nut-producing tree (Buttrick, 1915; Cameron, 2002). With the introduction of the chestnut blight fungus *Cryphonectria parasitica* at the beginning of the 20<sup>th</sup> century, most of the American chestnut trees were killed to the ground within a 30- to 40-year period (Anagnostakis, 1987). Substantial levels of resistance to *C. parasitica* (*Cp*) have been found in Asian species of *Castanea*, including Chinese chestnut, *C. mollissima*, and Japanese chestnut, *C. crenata* (Graves, 1950; Anagnostakis, 1992). Because of its cultural, ecological, and economic importance to the Appalachian Mountain region of the eastern U.S., The American Chestnut Foundation (TACF) and others in the forest genetics community have pursued a backcross breeding program to introgress *Cp* resistance from Chinese chestnut into American chestnut (Burnham, 1981, 1987). Late generation backcross trees now are being tested in different regions of the U.S. for resistance to *Cp* as part of an overall effort to reintroduce the American chestnut to the forests of the eastern U.S. (Hebard, 2005; Diskin et al., 2006).

At the same time, another introduced pathogen, *Phytophthora cinnamomi*, is limiting the range where the *Cp*-resistant American chestnut trees can be planted and grown. This pathogen causes *Phytophthora* root rot, also known as ink disease, and is prevalent in many of the soils in forests of the southern U.S., including those at the lower elevations of the Appalachian Mountains. American chestnut appears to have no resistance to *P. cinnamomi* (*Pc*) while Chinese chestnut and other Asian chestnut species are known to be *Pc* resistant (Crandall et al., 1945). Recognizing the threat associated with *P. cinnamomi*, James and Jeffers at Clemson University initiated a program in 2004 to evaluate hybrid chestnut seedlings for resistance to *Pc* at the Chestnut Return Farm in the piedmont region of South Carolina, USA (Jeffers et al., 2009; James 2011a, b). The goal was to find hybrid seedlings that could survive infection by *P. cinnamomi* and then test the survivors for resistance to *C. parasitica*. Their results suggested that the screening method was effective at detecting *Pc* resistance but only a small percentage of the test seedlings carried this resistance (Jeffers et al., 2012). Together this information clearly suggests that resistance to *Pc* can be improved with breeding. Resistance to both *Cp* and *Pc* will be required for successful American chestnut restoration in the southern portion of its original range.

To meet the challenge of producing *Cp* and *Pc* resistant materials, TACF has begun to incorporate *Pc* resistance screening into its breeding program using the evaluation method and facility described previously (Jeffers et al., 2009, 2012). In parallel, utilizing the backcross families being screened for *Cp*, we have begun a research project to genetically map and characterize the gene(s) in Chinese chestnut that control *Pc* resistance. These families were developed from crosses of American chestnuts with two Chinese chestnut cultivars, ‘Mahogany’ and ‘Nanking’. Having genetic markers for the resistance-conferring regions of the Chinese chestnut genome should expedite the selection of resistant seedlings and enable a more rapid deployment of resistant hybrid American chestnut trees in the restoration programs. Initial results in this mapping research are presented here.

### MATERIALS AND METHODS

#### Plant Material

Several mapping populations segregating for resistance to *P. cinnamomi* were available for genetic analysis. These include: 1) a progeny set of 48 individuals AdairKY1 × GL158, an interspecific BC<sub>1</sub> cross between an American chestnut accession

from Adair County Kentucky and an F<sub>1</sub> Chinese/American hybrid, GL158, derived from crossing GR12, a ramet of Nanking (maternal genotype) and an American chestnut tree Am33; and 2) two BC<sub>1</sub> crosses HB1 (KY115 × AD88) and HB2 (KY115 × AD98) and a BC<sub>4</sub> cross MK5 (PA Haun Row 1 Tree 18 x BG363) sharing the same source of resistance ('Mahogany'). Seeds for these mapping populations were generated by controlled pollination of trees maintained at the TACF Meadowview Research Farms in Virginia and by the Pennsylvania and Kentucky Chapters of TACF.

### **Phenotyping Procedure**

Phenotyping for resistance to *P. cinnamomi* was conducted at the Chestnut Return Farm, Seneca, SC in 2008 for the AdairKY1 × GL158 cross and in 2011 for the other three crosses (HB1, HB2, and MK5). Evaluating resistance to *Pc* followed a protocol developed by Jeffers and James that has been used consistently for nine years (Jeffers et al., 2009, 2012; James 2011a, b). Briefly, stratified seeds were planted outdoors in 570-L plastic tubs containing a soilless container mix using a randomized block planting design. American (susceptible) chestnut and Chinese (resistant) chestnut controls were planted randomly in each tub. After seedlings grew for 12 weeks, the soil was infested with a mixture of two isolates of *Pc* previously recovered from diseased chestnut trees at the study site. Inoculum was grown on vermiculite moistened with V8 Juice broth in the Jeffers laboratory at Clemson University.

### **Phenotype Scoring**

Evaluation of disease severity was based on visual examination of the roots on individual seedlings in December or January after plants were dormant. Four symptom severity classes were recognized: class 0 – roots healthy and no evidence of infection, class 1 – root rot symptoms on any of the feeder roots, class 2 – root rot symptoms on the tap root or severe root rot on the feeder roots, and class 3 – seedling dead (Jeffers et al., 2009). However, in 2008, the AdairKY1 × GL158 individuals also were scored based on above-ground symptoms on the seedlings at the end of the growing season (September). With this method, three symptom severity classes were recognized based on visual inspection of seedlings: class 1 – healthy, seedling with all or most of its leaves and no visual symptoms; class 2 – symptomatic, seedling alive but leaves had dropped prematurely; class 3 – seedling dead. Out of 48 seedlings in the AdairKY1 × GL158 cross, 27 appeared healthy, four were symptomatic and 17 were dead. In 2011, a total 1369 seedlings from 45 advanced generation crosses (B×F<sub>1</sub>, where x=1 to 4) were evaluated using the standard root evaluation method. Using these data, four crosses were selected for study – including DNA extraction, genotyping and mapping.

### **DNA Extractions and Genotyping**

For DNA extraction, leaves were collected before inoculation and DNA was extracted as described in Kubisiak et al. (2012). In total, 203 SNP markers were scored in the AdairKY1 × GL158 cross as described previously (Kubisiak et al., 2012; Olukolu et al., 2012). The SSR genotypes for the HB1 and HB2 crosses were determined using electrophoresis of radioactively labeled DNA fragments on polyacrylamide sequencing gels and autoradiography (Zhebentyayeva et al., 2003).

### **Mapping Analyses**

For QTL mapping in the AdairKY1 × GL158 cross, we utilized PLABQTL v1.2 (Utz and Melchinger, 1996) after using JoinMap v4.0 (van Ooijen, 2006) to create a low-density genetic map consisting of 203 SNPs on 12 linkage groups. For QTL analysis in the HB1 and HB2 cross, we used JoinMap v4.0 for linkage map construction and MapQTL5 for QTL mapping (van Ooijen, 2004).

## **RESULTS AND DISCUSSION**

Initial mapping of resistance to *P. cinnamomi* in a BC<sub>1</sub> family between a resistant

Chinese/American hybrid (GL158) and an American chestnut accession (AdairKY1) revealed significant QTLs only on LG\_E. These QTLs are depicted in Figure 1 as a locus (LOD 5.39) at the bottom-half of the LG\_E, resulting from segregation of GL158 alleles that explains approximately 40% of the phenotypic variation and a locus (LOD 4.42) toward the center of LG\_E, resulting from segregation of Adair KY1 alleles that explains approximately 34% of the phenotypic variation.

As these initial results were based on a very limited number of progeny, it was necessary to develop and evaluate much larger populations to verify these results. As part of TACF's breeding program for *Cp* resistance, 45 backcross progenies issued from resistant Chinese parents (mainly 'Mahogany' background) were available for *Pc* resistance testing in 2011. In addition, crosses HB1 and HB2 were bred specifically for this purpose. Based on root rot severity, resistant individuals (classes 0, 1, and 2) were detected in eight crosses (Table 1). Four crosses with the highest survival rate were selected for DNA extraction and genotyping. In particular, we were interested in the HB1, HB2, and MK5 crosses because they exhibited reasonable segregation for resistance as scored using above-ground symptoms compared with those employed for our initial mapping cross AdairKY1 × GL158.

An initial mapping analysis (Fig. 2) was performed utilizing 22 SSR markers that span LG\_E of the Chinese consensus map (Kubisiak et al., 2012) and a limited number of progeny, 55 and 47 plants in HB1 and HB2 crosses, respectively. A summary of the marker statistics used for the map construction is presented in Table 2. Although interval mapping with the maximum-likelihood algorithm may produce unreliable results when applied to classification data such as disease scores (van Ooijen, 2004), Kruskal-Wallis tests also indicated the presence of a QTL on LG\_E (Fig. 3), validating previous results for AdairKY1 × GL158. However, this work needs further verification with greater numbers of progeny and markers representing all linkage groups. Toward this end, we are evaluating *Pc* resistance and marker genotyping additional progeny in these crosses at the Chestnut Return Farm in 2012 using both the above-ground and below-ground scoring procedures.

Additionally in 2012, we are testing expanded populations of the HB2 cross (181 individuals) and including crosses derived from the resistant Chinese chestnut cultivar Nanking, the source of resistance in the AdairKY1 × GL158 cross (Table 3). Future analyses are expected to verify and delimit the resistance locus/loci on LG\_E. This will include a full genome scan for QTLs in these expanded crosses to determine if resistance is limited to LG\_E or if there is evidence of other resistance loci in the genome. Resistance mapping in different crosses having different donors of resistance also should allow us to determine the extent of the genome of Chinese chestnut contributing to resistance to *P. cinnamomi*.

## CONCLUSIONS

Our initial QTL mapping efforts with hybrid plant material derived from two Chinese chestnut sources ('Mahogany' and 'Nanking') support the hypothesis of a limited number of genomic regions underlying resistance to *P. cinnamomi*. Moreover, these two lineages of hybrid material might share resistant haplotypes located on LG\_E. Genetic positioning of the QTL(s) for resistance to *P. cinnamomi* will enable pyramiding of resistance to the two major chestnut pathogens (*C. parasitica* and *P. cinnamomi*), which is critical for American chestnut restoration in the southeastern U.S.

## ACKNOWLEDGEMENTS

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## **Tables**

Table 1. Advanced generation chestnut crosses selected for genotyping based on evaluation of Phytophthora root rot severity in 2011 (DNA was extracted from crosses in bold; crosses with stars were genotyped).

Cross code	Number plants	Symptom severity classes				Cross type	Female parent	Male parent
		0	1	2	3			
AM (control)	19	0	0	2	17	American	n/a	n/a
CHN (control)	18	13	5	0	0	Chinese	n/a	n/a
C1	54	1	4	4	45	B4F1	TedFarmA	SC342
C3	52	0	5	16	31	B4F1	TedFarmA	BG376
C7	46	0	1	2	43	B4F1	TedFarmB	BG37
<b>HB1*</b>	55	0	2	15	37	B1F1	KY115	AD88
<b>HB2*</b>	47	1	5	21	20	B1F1	KY115	AD98
IN2	59	0	6	1	51	B3F2	IW2×CL50	BG90
<b>MK5</b>	63	1	14	11	36	B4F1	HaunR1T18	BG363
OH1	38	0	3	1	34	B4F1	Ohio#6	BG310

Table 2. Summary of SSR marker statistics for the HB1 and HB2 crosses.

SSR markers	HB1	HB2
Screened	22	22
Unclear	2	2
Monomorphic	6	3
Mapped	13	12

Table 3. Extended mapping populations ongoing in 2012 and planned for 2013 for *Phytophthora cinnamomi* resistance phenotyping, genotyping, and mapping.

Evaluation year	Hybrid crosses	Number of plants	Source of resistance
2012	HB2	181	Mahogany
	NK1+NK2	113	Nanking
	NK3	39	Nanking
2013	HB2	250 (seeds)	Mahogany

## Figures

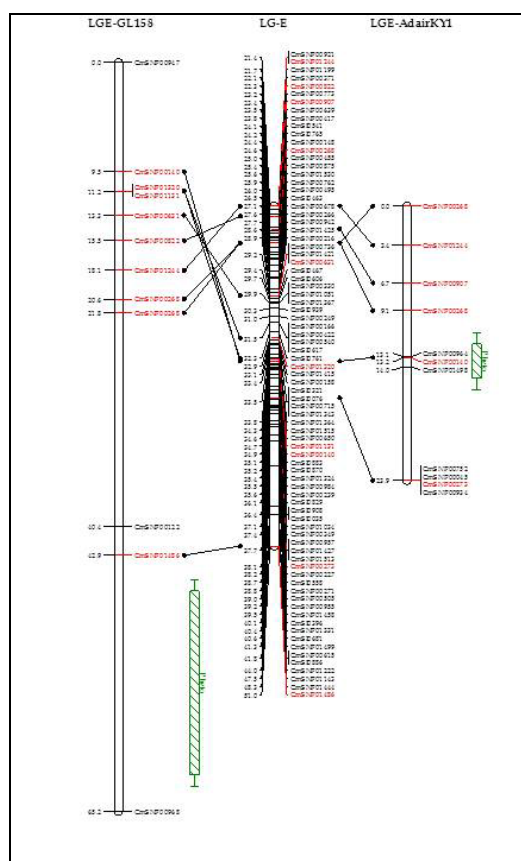


Fig. 1. QTL map of resistance to *Phytophthora cinnamomi* in progeny of a BC<sub>1</sub> cross (AdairKY1 (American) × GL158 (hybrid)). A portion of the consensus Chinese chestnut LG\_E is depicted in the middle; LG\_E segregating from GL158 and AdairKY1 are depicted on the left and right, respectively. The QTL intervals on the parental maps are depicted by green, hatched boxes. The significant threshold LOD scores 2.76 and 2.90 for AdairKY1 and GL158, respectively, were calculated based on 1,000 permutations at  $P \leq 0.05$ .

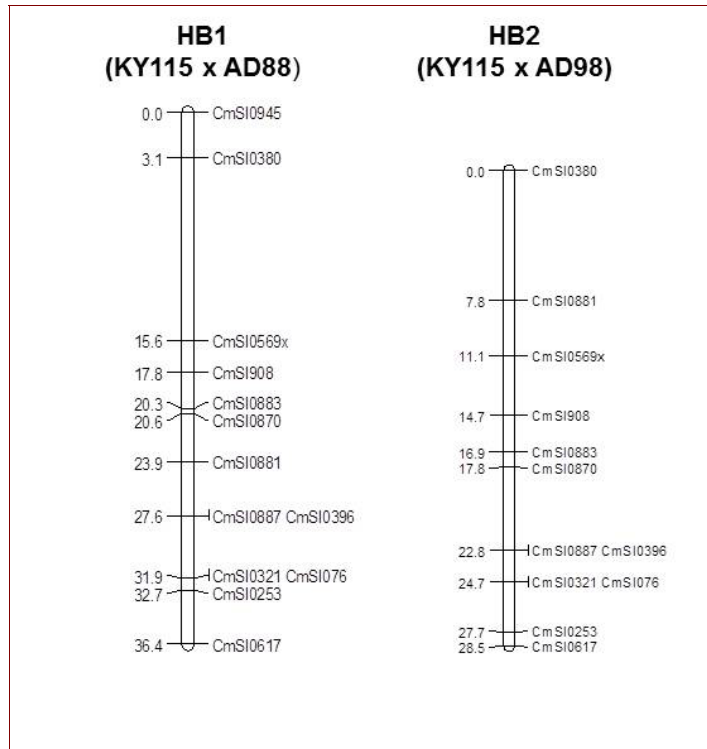


Fig. 2. Initial local maps of LG\_E in the HB1 and HB2 crosses using SSR markers mapped on the Chinese chestnut consensus map (Kubisiak et al., 2012). Linkage groups were established at minimum LOD score of 2.0.

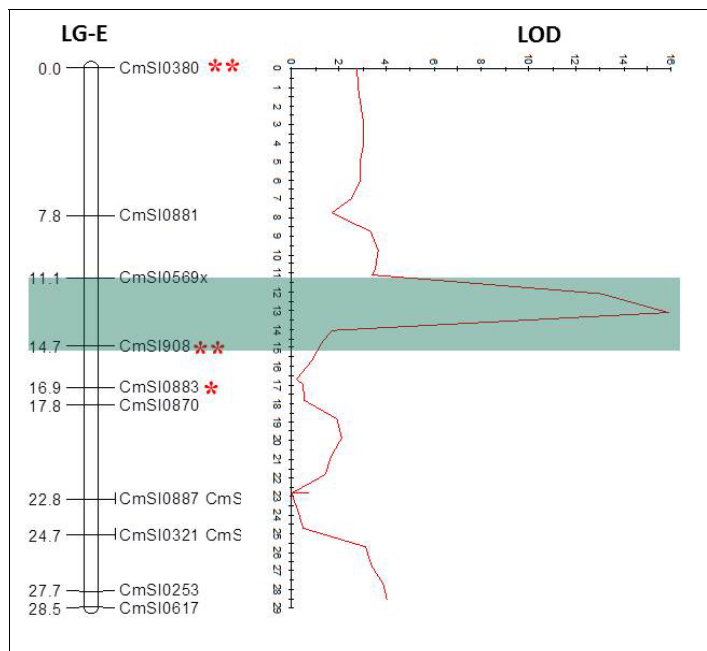


Fig. 3. Initial MQM QTL detection composite interval mapping results and the Kruskal-Wallis test-data for LG\_E of the HB2 cross. Markers associated with resistance to *Phytophthora cinnamomi* are significant at  $P \leq 0.1$  (\*) and  $P \leq 0.05$  (\*\*).