Final Report for "Mapping of resistance to *Phytophthora cinnamomi* (*Pc*) in interspecific American/ Chinese chestnut populations"

Principal Investigator: *Tetyana N. Zhebentyayeva,*
Genomics & Computational Biology Laboratory
Office 310 Biosystems Research Complex
105 Collings Street
Clemson, SC 29634
Tel: 864-656-4292
Email: tzhebe@clemson.edu

Co-Principal Investigators:
C. Dana Nelson, Southern Institute of Forest Genetics, Southern Research Station, USDA Forest Service, and Visiting Scientist, Department of Forestry, University of Kentucky
Albert G. Abbott, Forest Health Research and Education Center, University of Kentucky Department of Forestry, University of Kentucky
Christopher Saski, Laboratory for Genomics & Computational Biology and Department of Genetics & Biochemistry, Clemson University

Collaborating Scientists:
Stephen N. Jeffers, College of Agriculture, Forestry and Life Sciences, Clemson University
Joseph B. James, Chestnut Return Farm
Frederick V. Hebard, The American Chestnut Foundation
Laura L. Georgi, The American Chestnut Foundation
Paul H. Sisco, The American Chestnut Foundation

Objectives: **1)** construct a genome-wide genetic linkage map for the KY115 x AD98 cross (HB2-2014 in Table 1) using the Genotyping-by-Sequencing (GBS) approach and **2)** delineate and identify DNA sequence-based markers for the genomic intervals that contain the QTLs for resistance to Pc introgressed from 'Mahogany'.

Summary

Phytophthora root rot, caused by *Phytophthora cinnamomi* (*Pc*) and also called ink disease, is one of the major factors affecting restoration of American chestnut (*Castanea dentata*) in the southeastern U.S. Due to a lack of reproductive barriers, introducing resistance to *Pc* from Chinese chestnut (*C. mollissima*) into American chestnut is a feasible task for molecular breeding. To identify genomic regions in Chinese chestnut controlling resistance to *Pc* in interspecific BC_1F_1 progenies, we used a traditional approach—quantitative trait locus (QTL) analysis in bi-parental populations based on fine-scale linkage maps. Using genotyping-by-sequencing (*Pst*1-*Msp*1 double digestion, Illumina paired-end sequencing), we generated linkage maps for the HB2 (KY115 × AD98) cross consisting of 237 individuals segregating for severity of root rot symptoms.

Parental female and male maps were composed of 626 and 497 non-redundant and non-distorted ($P \leq 0.05$) SNP markers, respectively. The markers were organized into 12 linkage groups covering genetic distances of 581.2 cM on the KY115 map and 691.5 cM on the AD98 map. Using sequenced marker information, the HB2 linkage groups were anchored to the *C. mollissima* genome v1.1 scaffolds and oriented against the *C. mollissima* reference map. Three significant QTLs (LG_A, LG_E and LG_K) were detected for the KY115 parent, which was derived from the Chinese chestnut cultivar 'Mahogany'. An additional QTL for resistance on LG_A was detected in susceptible parent AD98.

Background and significance

Restoration of American chestnut in the southeast requires pyramiding resistance to both major fungal diseases caused by *Cryphonectria parasitica* (*Cp)* and *Phytophthora cinnamomi (Pc)*. Substantial levels of resistance to both pathogens have been found in Asian species of *Castanea*, in particular Chinese chestnut (*C. mollissima*) and Japanese chestnut (*C. crenata*). An effective screening method for detecting *Pc* resistance was established as a result of collaborative efforts among the USDA Forest Service, Forest Health Research and Education Center (FHREC), The American Chestnut Foundation (TACF), Clemson University and The Chestnut Return Farm (Jeffers et al. 2009; Jeffers et al., 2012). In parallel, a study was initiated to determine the genetic basis for *Pc* resistance and to predict *Pc* resistance in advanced breeding material.

Development of a set of transcriptome-based markers and construction of the reference *Castanea* genetic maps improved the delineation of the genomic regions underlying three QTLs for resistance to *Cp* (Kubisiak et al., 2013). Identification of markers associated with three QTL intervals for resistance to *Cp* (*Cbr1*, *Cbr2*, and *Cbr3*) was initiated through whole-genome resequencing of resistant vs. susceptible individuals that originated from the F_2 segregating cross (A.G. Abbott, unpublished). A number of SSR and SNP markers have been generated in support of the TACF backcross breeding program for *Cp* resistance. Unfortunately, identification of genetic regions controlling resistance to *Pc* is far from being implemented in breeding. So, the **ultimate goal** of this project is to address the construction of a genetic linkage genetic map and delineate the genomic regions conferring resistance to *Pc* derived from four resistant genotypes - two from the species *C. mollissima* ('Mahogany', 'Nanking') and two from the species *C. crenata* ('Morrow Mountain', 'Fort Defiance'). Delineation of genetic intervals conferring *Pc* resistance will facilitate the identification of markers associated with this important trait and their practical implementation in breeding for resistance to both chestnut pathogens, *Cp* and *Pc*. Due to limited funding available through this call for proposals, we narrowed our tasks and selected for study an extended BC_1F_1cross - KY115 × AD98 ('Mahogany' background) phenotyped for PRR resistance in 2014

(Table 1). Our **specific objectives** for 2015 were to: 1) construct a genome-wide genetic linkage map for the KY115 × AD98 cross (HB2-2014 in Table 1) using the Genotyping-by-Sequencing (GBS) approach and 2) delineate and identify DNA sequence-based markers for the genomic intervals that contain the QTLs for resistance to *Pc* introgressed from 'Mahogany'.

Plant material

The proposed study is based on hybrid plant material generated by TACF's Meadowview Research Farms (the HB2 cross) and by the Carolinas Chapter of TACF (all the other crosses) for evaluating resistance to *Pc*. Phenotyping for severity of root rot symptoms (PRR test) was done in 2011-2015 at the Chestnut Return Farm, Seneca, SC following protocol by J.B. James and S.N. Jeffers at (Jeffers et al, 2009; Jeffers et al, 2012). Typical appearance of plants in four phenotypic classes is shown in Fig.1. Since 2011, extended hybrid crosses were developed for statistical analysis, genetic mapping and QTL detection. These populations segregating for resistance to *Pc* are hybrid lineages derived from crosses of two resistant individuals of the Chinese chestnut (*C. mollissima),* 'Mahogany' and 'Nanking' with susceptible American accessions. Recently, a 3rd source of resistance, Japanese chestnut 'Morrow Mountain', was also included in the analysis (Table 1). Multiple year of phenotyping in 2011-2014 are available for genetic studies in the BC_1 cross HB2 (KY115 × AD98) having a combined progeny size of 684 individuals. Of these 237 individuals phenotyped in 2014 (deemed as HB2-2014 cross) were chosen to genotype and to analyze in the frame of this proposal.

DNA extractions, GBS library constructions and sequencing

Young leaves were harvested before inoculation. Genomic DNA was extracted from 100 mg of leaf tissue using a modified CTAB protocol by Kubisiak et al. (2013). DNA quality was accessed on 1% agarose gels. Chestnut DNA was quantified with Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen) against a λ standard DNA dilution series with a Synergy H1 microplate reader (BioTek, Winooski, VT). A test digestion for 26 random samples was done with *Hind*III restrictase followed by fragment resolution on 1% agarose gels. High throughput SNP genotyping was conducted using methods, barcodes, adapters, and primers of Elshire et al. (2011). In brief, each DNA extract (100 ng) was double digested for 3 hours with restriction enzymes *Pst*I and *Msp*I and indexed with individual barcodes using T4-DNA ligase (all enzymes from New England Biolabs, Ipswitch MA). To create a saturated dataset for SNP calling in segregating material, DNA of each parent was sequenced 3 times. Ligation products from each DNA extract were pooled (48 individuals per pool) and purified using QIAquick PCR purification kit (Qiagen, Valencia, CA). The amplified 48-plex libraries were checked using 2100Bioanalyzer (Agilent Technologies, Santa Clara, CA) for fragment size distribution and presence of dimer picks. Fragments of 200-700 bp were twice (0.4x and 0.85x) size-

selected with Agencourt AMPure XP magnetic beads (Beckman Coulter, Inc). Pair-end sequencing 2 x 125 bp was done using llumina HiSeq 2500 sequencer at Hollings Cancer Center (Medical University of South Carolina, Charleston, SC.

Data processing and bioinformatic pipeline

Raw GBS data were processed using the *Stacks* software package, version 1.32 (Catchen at al., 2011). Quality filtering of raw reads and demultiplexing based on individual barcode was conducted using '*process_radtags'* command. Reads were aligned against the reference *Castanea mollissima* v1.1 genome scaffolds using GSNAP short reads aligner version 2015-09-29 (Wu and Nacu, 2010). Files with alignment (in bam format) were sorted with SAMtools v.1.2 (Li et al., 2009) and indexed with picard-tools-1.74 (http://broadinstitute.github.io/picard/). Alignments with mapping quality > = 2 were used for SNP calling. An SNP discovery and genotyping was done with Stacks commands '*ref_map*' and '*genotypes*' with the following options: CP (cross pollinator full-sib population) population, depth of reads 15x, less than 10% of missing data (genotypes present in at least 215 individuals),'joinmap' output file format.

Linkage map construction

The linkage mapping was conducted using JoinMap 4.1 software (Van Ooijen, 2006) with the CP (cross pollinator full-sib population) population option following the two-way pseudo-testcross mapping strategy for heterogeneously heterozygous species (Grattapaglia and Sederoff, 1994). Only markers segregating 1:1 (<*lmxll*>, <*nnxnp*> configurations) and 1:1:1:1:1 (<*abxcd*> configuration) were used for mapping. Segregation distortion of individual markers was calculated using the χ^2 test and markers with significant segregation distortion ($p \leq 0.05$) were excluded using JoinMap function. Additionally, highly similar markers (>0.95) were excluded from the data set to reduce calculation time. Two parental maps were established at independence LOD (logarithm-of-odds) score threshold of 6.0 and 4.0 for the female and male parents respectively. Marker order and distances within linkage groups were calculated using the Kosambi mapping function (Kosambi, 1944) with default settings: a recombination frequency less than 0.40, a minimum jump LOD score of 1, and the goodness-of-fit jump threshold of 5.0. Correspondence between parental maps was identified using markers polymorphic in both maps (<abxcd> configuration). The HB2 linkage groups were '*in silico'* assigned and oriented against the Chinese chestnut reference map by Kubisiak et al (2013) based on interpolation marker positions on both maps via anchored genomic scaffolds. Briefly, sequence informative SSR/SNPs markers from Chinese chestnut map first were assigned to *Castanea mollissima* v1.1 scaffolds using BLAST® (Basic Local Alignment Search Tool) (Altschul et al. 1990). Data on assignment of the GBS markers to *C. mollissima* v1.1 scaffolds were extracted from catalog of the GBS tags, one of the Stacks output files. The maps were drawn using MapChart v2.3 (Voorrips, 2002).

QTL detection

QTL analysis was performed using Kruskal–Wallis, interval mapping (IM) and multiple-QTL mapping (MQM) analyses implemented into MapQTL6.0 (Van Ooijen, 2009). The presence of QTLs between markers within each interval was ascertained by the likelihood ratio test performed at every cM along the linkage group. Genome-wise LOD score threshold was determined on the basis of 1,000 permutations. A LOD threshold of 2.8 corresponding to a genome-wide significance level of $P \leq 0.05$ was chosen. The 'Automatic Cofactor Selection' tool was used iteratively to identify the strongest marker cofactors on each linkage group for each trait. Resulting cofactors were included in the search for QTLs that exceeded the LOD significance threshold. The position of each QTL was estimated, along with its additive and dominance effects, the mean degree of dominance, and the percentage of total variance of the trait explained by the QTL. LOD curves were plotted with the MQM QTL detection algorithm and exported using MapQTL option.

Results

GBS data processing statistics and genotyping

A total of 1,880 million reads were produced for HB2-2014 individuals by the HiSeq2100 platform (Table 2). Of these, 96.5% of reads (1,815 million) were retained after sequential quality filtering and sequence trimming. Eight individuals were excluded from the dataset due to a low number of reads (less than 1 millions). Parental genotypes were sequenced at a high depth. In total, 48.0 and 37.4 millions reads were produced for female KY115 and male AD98 parents. The average clean reads for each progeny was 7.5 million while density of reads for parents was about 5-6 times higher – 47.4 and 36.9 million reads for KY115 and AD98 respectively. A catalogue containing 82,662 SNP loci was generated from parental data sets and used as reference for SNP genotyping in the mapping population. In total, 19,654 SNP markers were identified across all the samples and used for filtering out loci with missing data.

Saturated the HB2-2014 linkage map

A subset of 3,641 SNPs present in more than 90% of the progeny was used in linkage mapping. Of these, 238 and 134 loci in <*efxeg*> and <*hkxhk*> configurations were deleted. Retained loci were polymorphic either in female KY115 (2,657 SNPs) or in male AD98 (569 SNPs) or in both parents (43 SNPs in <abxcd> configuration). Due to the removal of 8 individuals with more than 10% of missing data, the effective progeny size was 229 individuals. Altogether twelve linkage groups were established at LOD 6.0 and 4.0 on female KY115 and male AD98 maps (Fig.2). Only 13 loci in parental datasets

(0.03% of total markers) remained ungrouped. Global mapping statistics for female and male maps presented in Table 3. The 4-times fewer loci in the AD98 dataset compared with that in KY115 (612 vs. 2700) could be explained by the fact that KY115 was a Chinese/American hybrid with more differences between alleles and by the fact that the reference map used to identify loci was created from Chinese chestnut rather than American chestnut.

In JoinMap, markers were first filtered for segregation distortion ($P \leq 0.05$) and then only one marker per genetic position was retained during iterative mapping procedure. Overall in the HB2-2014 population, observed segregation of 10.84% markers (292 out of 2693) deviated from expected Mendelian ratios with majority of distorted loci in interspecific American x Chinese hybrid KY115 (67.46%). In KY115, extended blocks of segregation-distorted markers (156 loci) were found across almost the entire LG_I. Most of the loci were homozygous and distorted in favor of American chestnut, a recurrent parent in our BC_1 cross. Large contiguous block of segregation-distorted markers was also detected in the bottom part of LG_A. In this block a heterospecific allele composition prevailed over homozygous genotypes.

Linkage maps were composed of 626 and 491 markers in the female and male parents, covered 581.2 cM and 691.6 cM of genetic distances with average marker density 0.93 and 1.41 cM per marker respectively (Table 3, Fig.2). Due to the removal of distorted markers, the smallest group LG_I in KY115 was composed of 5 loci and covered 15.1 cM with highest average marker density 3.02 cM per loci. Parental maps were oriented and assigned to the reference Chinese chestnut map by Kubisiak et al. (2013) as described in Methods. Initially we used 26 retained SNP loci in <abxcd> configuration, which are polymorphic and heterozygous in both parents, to orient the KY115 and AD98 maps to each other. Additional anchor points were provided by '*in silico*' assigning the HB2 linkage groups to Chinese chestnut map via genome scaffolds. Altogether, 244 SNP parental loci share the *C. mollissima* scaffolds harboring sequence-informative markers from Chinese chestnut map. Thus, robust parental maps for female KY115 and male AD98 parents were created providing a solid foundation for QTL analysis in our project.

Genomic regions underlying resistance to *Phytophthora cinnamomi*

Segregation of the HB2 hybrids family phenotyped for resistance to *Pc* during four consecutive years 2011 through 2014 is presented in Table 5. For comparison we have phenotypic data for NK4-2014 cross representing another Chinese chestnut source of the *Pc* resistance, cultivar Nanking. Based on a multiple year data, crosses HB2-2014 (237 individuals) and NK4-2014 (318 individuals) were selected for high-throughput genotyping by sequencing due to a high number of individuals and segregation ratio that could be explained by 2-3 genomic regions (our working hypothesis). In 2014 the HB2 progeny segregated for resistance to *Pc* in a ratio 1:1/alive: dead ($\chi 2 = 1.52$, $p \leq 0.05$) that

was consistent with 1:1 ratio observed in the NK4-2014 cross has been phenotyped simultaneously ($\chi 2$= 0.3, $p \leq 0.05$). Parental KY115 and AD58 maps were separately used to test if QTLs can be detected using interval mapping, Kruskal-Wallis test and multiple-QTL mapping procedures implemented into JoinMap6.0. Multiple-QTL mapping identified three significant QTL for resistance to *Pc* on female KY115 map, on linkage groups A, E, and K (Fig. 3). They explained 9.9%, 13.8% and 19.1% of the phenotypic variation (Table 5). These QTLs were named accordingly as *qPc*_A.1, *qPc*_E.1, and *qPc*_K.1 for KY115 parent. The results of QTL mapping using alternative statistical approaches of nonparametric analysis and interval mapping were similar and consistent. Surprisingly, a QTL interval for *Pc* resistance was also detected on LG_A on the AD98 map (*qPc*_A.2), which explains 11.8% of phenotypic variation with negative effect on the trait (Table 5). Important to notice, the QTL intervals on LG_A on KY115 and AD98 maps reside in an overlapping genomic region flanked by the reference makers CmSNP00552 and CmSNP00743 on the female and CmSNP00160 and CmSNP00473 on the male maps.

Discussion

Parental KY115 and AD98 maps and distorted markers

Using a high-throughput GBS approach we genotyped HB2-2014 cross segregating for resistance to *Pc* and generated two maps for female parent KY115, resistant interspecific American × Chinese chestnut hybrid, and male parent AD98, susceptible American chestnut genotype. Maps spanning 581.2 cM and 691.6 cM respectively were oriented against Chinese chestnut map providing a solid foundation for delineation QTL intervals between crosses derived from Chinese chestnut Mahogany and other sources of resistance. Extensive region of distortion favoring heterospecific alleles and covering nearly the entire length of a linkage groups was reported in interspecific BC_1 crosses in poplar (Yin et al. 2004). The authors explained their observations by positive selection for the introgressing alleles. In out study, positive selection also may be a case for explaining distortion in favor of heterozygous alleles for LG_A. However, prevalence of homozygous alleles of recurrent parent (American chestnut) revealed on the LG_I can be attributed to other factor such as pollen-pistil incompatibility or other type of prezygotic barriers. Recently, Bodenes et al (2012) reported a genetic map for an interspecific BC_1 oak cross with a missing linkage group due to segregation distortion of loci along the entire linkage group in favor of homozygous alleles. This was attributed to reproductive barriers between related oak species. Segregation distortion is a common phenomenon observed during map construction. A number of non-biological factors such as sampling or genotyping errors may cause a deviation from expected ratios. In this study, we have chosen an option, in which only markers following Mendelian segregations were used in mapping. However, the exclusion of such markers could bias the data and result in a loss of some important information on genomic region controlling biological phenomena such

as self-incompatibility, sex-related traits or tree vitality. Using the same SNP dataset and maximum-likelihood (ML) mapping, we have constructed saturated parental maps for use for assembly of the chestnut genome scaffolds. The ML map will be also used to verify that significant information was not lost because of removing distorted markers.

QTL mapping resistance to Pc

Since development of standardized protocol for Phytophthora root rot phenotyping (PRR test) (Jeffers et al. 2009; Jeffers et al., 2012) we undertook several efforts to map the *Pc* resistance in interspecific American x Chinese chestnut populations. Initially a low-density map was constructed for 48 individuals issued from a cross of AdairKY1 × GL158. GL158 was an F1 tree whose Chinese parent was the cultivar 'Nanking'. A genome-wide low-resolution map composed of 203 SNPs was constructed, and a strong QTL signal was detected on LG_E (Kubisiak, 2010; Olukolu et al., 2012). Using local SSR-based genetic maps, the QTL on LG_E was confirmed in half sib crosses NK1+NK2 (Nanking background) and HB2-2011 cross (Mahogany background) (Zhebentyayeva et al. 2014). Thus, the QTL signal on LG_E (q*Pc*_E.1) detected in this analysis of the HB2-2014 cross is in agreement with previous observations.

We identified 3 QTL signals on LG_A, LG_E and LG_K of the KY115 map of resistant parents and 1 QTL on LG_A of AD98 map of susceptible parent. These QTLs can cumulatively explain 54.6% of phenotypic variation. However, no major loci (explaining >20%) were detected. The fact that these QTLs in HB2 cross do not independently have a higher contribution to *Pc* resistance is not unexpected. Resistance to *Pc* is most likely under control of complex dynamic gene networks that are activated at different stages of infection. Also, it is not unusual for the susceptible parent to carry QTLs for disease resistance. Alleles for increased resistance in the susceptible parent were found for *Phytophthora infestans* in tomato (Johnson et al. 2012), *P. soja* in soybean (Lee et al., 2013), *P. capsici* in pepper (Truong et al. 2012), and *P. palmivora* in cacao (Crouzillat et al., 2000).

As indicated by the performance of the HB2 cross over multiple years 2011-2014, resistance to *Pc* is significantly affected by environmental factors. This resulted in variations in the proportions of surviving plants (Table 4). Genotype × environment interactions are a common feature of genetic studies of resistance. To evaluate stability QTLs across multiple years, we are looking forward for genotyping HB2-2012 and HB2-2013 populations. Genotyping of other crosses derived from other sources of resistance (Table 1) will allow evaluation of the stability of the QTLs in different genetic backgrounds.

Conclusive Remark

An initial budget of this proposal covered only genotyping for HB2-2012 cross (179 individuals) using unidirectional Illumina sequencing (1x101 bp). However, we found additional sources of funding that allowed for an increased number of individuals for genotyping the HB2-2014 cross (237 individuals). Also, additional funding allowed more extensive sequencing of GBS libraries (2 additional lanes on HiSeq2100 Illumina instrument) using a paired-end sequencing (2x 125bp) instead of unidirectional sequencing. Thus, at no additional cost for TACF we generated more extensive SNP dataset for linkage mapping and QTL analysis of resistance *to Pc*. In parallel, we also constructed a saturated genetic map using maximum likelihood mapping procedure that allowed ordering of all distorted makers within linkage groups. The ML map generated for HB2-20154 cross will be used for improving of the *C. mollissima* genome assembly by John Carlson's lab at the Schatz Center for Tree Molecular Genetics (Penn State).

Results were presented at the following meetings:

1. Zhebentyayeva T, Perkins MT, Staton M, Jeffers S, James J, Sisco P, Hebard F, Georgi L, Olukolu B, Nelson CD, Abbott AG. Mapping of resistance to Phytophthora cinnamomi in interspecific American/Chinese and American/Japanese chestnut family hybrids. The NE-1333 Technical Committee Meeting on "Biological Improvement of Chestnut through Technologies that Address Management of the Species, its Pathogens and Pests", Hungry Mother State Park, Marion, VA September 10-12, 2015, Annual Meeting Minutes, p.30-31. (http://ecosystems.psu.edu/research/chestnut/meetings/crees-ne-projects/minutes-pdfs/2015-research-minutes)

2. Zhebentyayeva T, Perkins MT, Staton M, Jeffers S, James J, Sisco P, Hebard F, Georgi L, Olukolu B, Nelson CD, Abbott AG (2015). Mapping of resistance to *Phytophthora cinnamomi* (*Pc*) in interspecific American/ Chinese and American/Japanese chestnut hybrid families. The 33rd Southern Forest Tree Improvement Conference, June 8-11, 2015, Hot Springs, Arkansas (http://www.afrc.uamont.edu/sftic/)

3. Zhebentyayeva T, Georgi L, Jeffers CN, James J, Sisco P, Hebard FW, Saski CA, Staton M, Nelson CD, Abbott AG (2016) Development of a High-Density Genetic Map for QTL Analysis of Resistance to *Phytophthora cinnamomi* in an Interspecific American × Chinese Chestnut Population. The XXIV Plant & Animal Genome meeting, 8-13 January 2016, San Diego, California. Poster #1139 (https://pag.confex.com/pag/xxiv/webprogram/Paper21492.html)

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Bodénès C, Chancerel E, Gailing O, Vendramin GG, Bagnoli F, Durand J et al. (2012). Comparative mapping in the *Fagaceae* and beyond with EST-SSRs. BMC Plant Biol 12: 153.

Catchen JM, Amores A, Hohenlohe PA, Cresko WA, Postlethwait JH. 2011. Stacks: building and genotyping loci de novo from short-read sequences. G3 Genes Genomes Genetics 1: 171-182.

Crouzillat D, Phillips W, Fritz J, Petiard V (2000) Quantitative trait analysis in *Theobroma cacao* L. Using molecular markers: inheritance of polygenic resistance to *Phytophtora palmivora* in two related populations. Euphytica 114:25–36

Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchell SE (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLoS ONE 6, e19379.

Grattapaglia D, Sederoff R (1994) Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo‐testcross mapping strategy and RAPD markers. Genetics 137: 1121‐1137.

Jeffers SN, James JB, Sisco PH (2009) Screening for resistance to *Phytophthora cinnamomi* in hybrid seedlings of American chestnut. Pages 188-194 in: Proceedings of the Fourth Meeting of the International Union of Forest Research Organizations (IUFRO) Working Party S07.02.09: Phytophthoras in Forests & Natural Ecosystems. Goheen, E.M. and Frankel, S.J., tech. coords. Gen. Tech. Rep. PSW-GTR-221. US Dept. of Agriculture, Forest Service, Pacific Southwest Research Station. Albany, CA. 334 p.

Jeffers SN, Meadows IM, James JB, Sisco PH (2012) Resistance to *Phytophthora cinnamomi* among seedlings from backcross families of hybrid American chestnut. Pages 194-195 in: Proceedings of the Fourth International Workshop on the Genetics of Host-Parasite Interactions in Forestry: Disease and Insect Resistance in Forest Trees.

Johnson EB, Haggard JE, St.Clair DA (2012) Fractionation, Stability, and Isolate-Specificity of QTL for Resistance to *Phytophthora infestans* in Cultivated Tomato (*Solanum lycopersicum*). G3: Genes|Genomes|Genetics. (10):1145-1159. doi:10.1534/g3.112.003459.

Kosambi DD (1944) The estimation of the map distance from recombination values. Ann. Eugen. 12: 172-175.

Kubisiak TL. 2010. QTL analysis of *Phytophthora* resistance in a BC1 population. NE-1033 Annual Meeting Minutes, 2010, Maggie Valley, NC. p.18.

Kubisiak TL, Nelson CD, Staton ME, Zhebentyayeva T, Smith C, Olukolu B, Fang G-C, Anagnostakis S, Hebard FV, Wheeler N, Abbott AG, R.R. Sederoff RR (2013) A transcriptome-based genetic map of Chinese chestnut, (*Castanea mollissima*), and identification of regions of segmental homology with peach (*Prunus persica*). Tree Genetics & Genomes, 9: 557-571

Lee, S., Mian, M. A. R., McHale, L. K., Wang, H., Wijeratne, A. J., Sneller, C. H., & Dorrance, A. E. (2013). Novel quantitative trait loci for partial resistance to *Phytophthora sojae* in soybean PI 398841. TAG. Theoretical and Applied Genetics, 126(4), 1121–1132. http://doi.org/10.1007/s00122-013-2040-x

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. and 1000 Genome Project Data Processing Subgroup (2009) The Sequence alignment/map (SAM) format and SAMtools. Bioinformatics 25: 2078-2079.

Oblessuc, P.R., Baroni, R.M., Gracia, A.A. F., Chioratto, A.F., Carbonell, S.A. M., Camargo, L.E. A., and Benchimol, L.L.(2012). Mapping of angular leaf spot resistance QTL in common bean (*Phaseolus vulgaris* L.) under different environments. BMC Genet. 13: 50.

Olukolu, B.A., Nelson, C.D. and Abbott, A.G., 2012. Mapping resistance to Phytophthora cinnamomi in chestnut (Castanea sp.). In Proceedings of the Fourth International Workshop on the Genetics of Host–Parasite Interactions in Forestry: Disease and Insect Resistance in Forest Trees. July (Vol. 31, p. 177).

Truong, H. T. H., Kim, K. T., Kim, D. W., Kim, S., Chae, Y., Park, J. H., Oh, D. G., and Cho, M. C. 2012. Identification of isolate-specific resistance QTLs to *Phytophthora* root using an intraspecific recombinant inbred line population of pepper (*Capsicum annuum*). Plant Pathol. 61:48-56.

Van Ooijen JW (2006) JoinMap® 4, Software for the caluculation of genetic linkage maps in experimental populations. Kyazma B.V., Wageningen, Netherlands.

Van Ooijen JW (2009) MapQTL ® 6 Software for the mapping of quantitative trait loci in experimental populations of diploid species: Kyazma B.V., Wageningen, Netherlands.

Voorrips R (2002) MapChart: software for the graphical presentation of linkage maps and QTL. J. HereditMQM-AD98-1.emfy, 93: 77–78.

Wu TD, Nacu S (2010) Fast and SNP-tolerant detection of complex variants and splicing in short reads. Bioinformatics 26:873-881

Yin TM, DiFazio SP, Gunter LE, Riemenschneider D, Tuskan GA. Large-scale heterospecific segregation distortion in *Populus* revealed by a dense genetic map. Theor Appl Genet. 2004;109: 451–463.

Zhebentyayeva T, Staton M, Olukolu B, Chandra A, Jeffers S, James J, Sisco P, Hebard F, Georgi L, Nelson CD and Abbott AG (2014) Genetic and Genomic Resources for Mapping Resistance to Root Rot Disease (*Phytophthora cinnamomi*) in Chestnut. Acta Horticulturae (ISHS) 1019:263-270.

Tables and Figures

Table1. Interspecific hybrid populations developed for linkage analysis and QTL mapping of the resistance to root rot disease in chestnut

Hybrid population code-year	Total plants	Root rot symptoms severity classes 0	1	2	3	Type of family	Source of resistance
HB2-2011	41	1	3	20	17	BC1	
HB2-2012	179	0	2	122	55	BC1	
HB2-2013	230	0	18	146	66	BC1	
HB2-2014*	**237**	**0**	**3**	**106**	**128**	BC1	*C. mollissima* 'Mahogany'
JB1-2013	115	2	20	37	56	BC3	
JB1-2014	214	1	4	62	147	BC3	
MK5-2011	63	1	14	11	36	BC4	
NK1-2012	20	1	13	6	0	BC1	
NK2-2012	83	2	32	42	7	BC1	*C. mollissima* 'Nanking'
NK4-2014	318	2	17	135	164	BC1	
NK4-2015	151	in progress				BC1	
NK6-2015	227	in progress				F2	
MJ1-2014	76	0	1	22	53	BC1	*C. crenata* 'Morrow Mountain'
MJ1-2015	110	in progress				BC1	
MJ2-2015	31	in progress				BC1	

*HB2 phenotyped in 2014 and genotyped for this report

Table 2. GBS statistics for HB2-2014 individuals and parents

Tables anf Figures	**HB2-2014 individuals**	**Female KY115**	**Male AD98**
Total reads (mln)	1,880	48.0	37.4
Retained reads (mln)	1,815	47.4	36.9
Retained reads (%)	96.5	98.8	98.7
Reads/per individual (mln)	7.5	47.4	36.9
"Failed" (< 1 mln reads/ind)	8	-	-
Total SNPs (at P<0.05)	3,641	2700*	612*

* Polymorphic loci in input mapping f

Table3. Summary statistics of the HB2-2014 linkage map developed from GBS SNP markers.

Linkage group	Total SNPs available for mapping		Mapped SNP loci		Length (cM)		Average distances (cM)	
	KY115	*AD98*	*KY115*	*AD98*	*KY115*	*AD98*	*KY115*	*AD98*
A	439	56	46	51	44.8	89	0.97	1.75
B	281	62	69	39	61.6	55.9	0.89	1.43
C	188	62	58	48	52	59.7	0.9	1.24
D	167	41	35	40	51.4	63	1.47	1.58
E	239	59	74	51	41.4	55.1	0.56	1.08
F	268	63	85	55	66.1	56.7	0.78	1.03
G	146	48	31	24	59.5	35.9	1.92	1.5
H	222	58	42	45	28.7	51.9	0.68	1.15
I	161	57	5*	50	15.1	63.2	3.02	1.26
J	229	29	54	28	50.5	52.8	0.94	1.89
K	196	29	71	28	61.7	61.5	0.87	2.2
L	157	41	56	32	48.4	46.9	0.86	1.47
Total	2693	605	626	491	581.2	691.6	0.93	1.41

* 156 distorted markers deleted (P<0.05)

Table 4. Segregation ratio for resistance to *Pc* in HB2 and NK4 crosses phenotyped in -2011-2014

population code/year	Total plants	Root rot symptoms severity classes				segregation ratio	chi-squire test	p-value
		0	1	2	3			
HB2-2011	41	1	1	20	17	1:1	1.2	0.274
HB2-2012	179	0	3	122	55	2:1	0.57	0.459
HB2-2013	230	0	18	146	66	2:1	2.22	0.136
HB2-2014	237	0	3	108	128	1:1	1.52	0.217
NK4-2014	318	2	7	135	164	1:1	0.3	0.584

Table 5. Multiple-QTL mapping and Kruskal–Wallis analyses of QTL underlying *Pc* resistance in interspecific American x Chinese chestnut cross HB2-2014.

Parental map	QTL*	Linkage group	Peak LOD	Peak position (cM)	Cofactor (GBS locus)	PVE (%)	Effect	Kruskal-Wallis test (P)
KY115	*qPc*_A.1	LG_A	3.20	23.40	52208	9.9	0.23	0.005
KY115	*qPc*_E.1	LG_E	4.35	18.20	54410	13.8	0.27	0.0001
KY115	*qPc*_K.1	LG_K	5.92	18.80	28593	19.1	0.32	0.0001
AD98	*qPc*_A.2	LG_A	3.26	42.90	39959	11.8	-0.25	0.001

* Resistance QTLs (q) named as follows: the *Pc* abbreviation stands for pathogen causing disease (*Phytophthora cinnamomi*); the first letter refers to name of linkage group; the number is the QTL number on that linkage group.

Fig.1. PRR-test: phenotypic classes based on root rot symptoms (size and severity of lesions) used in this study

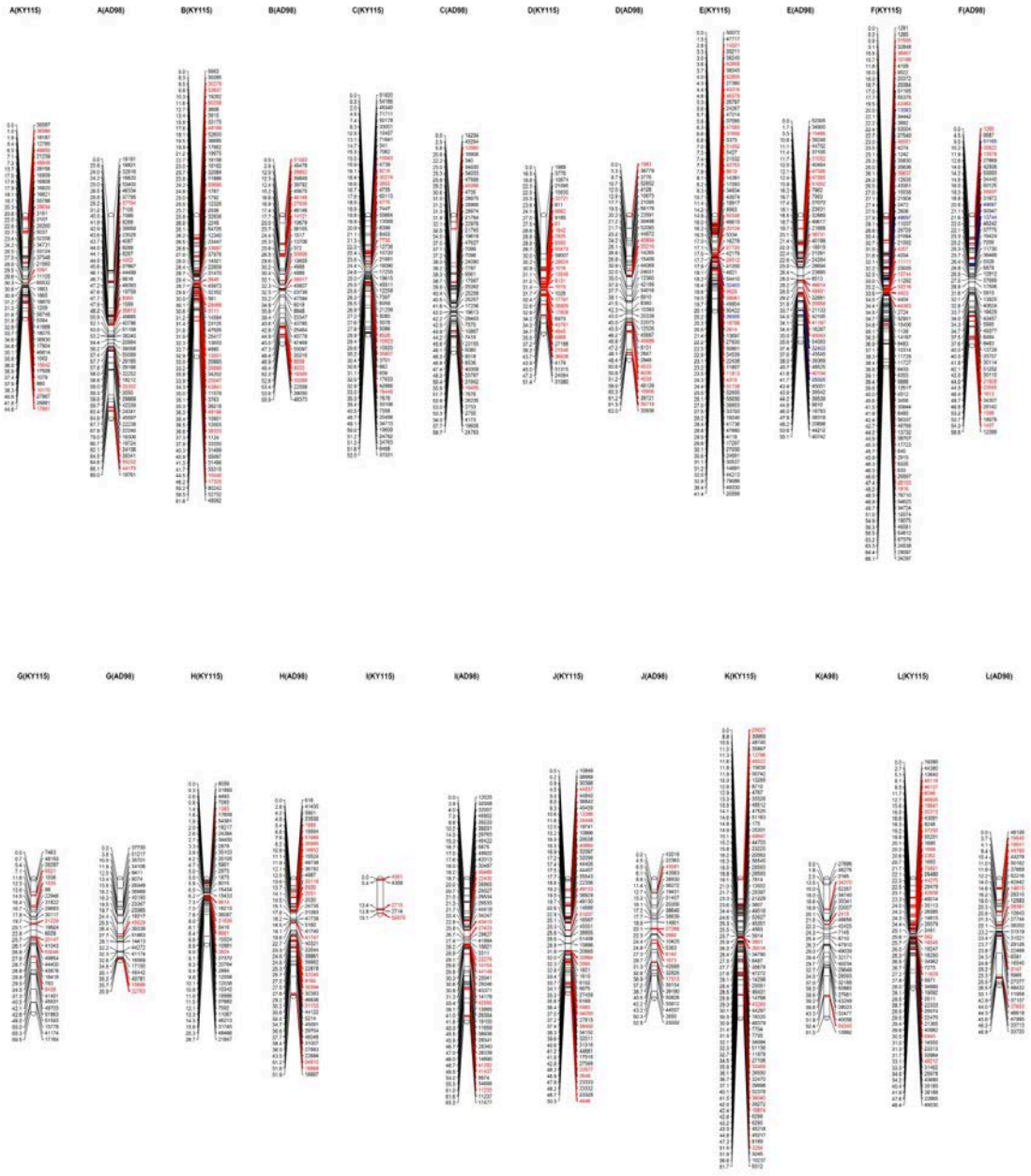
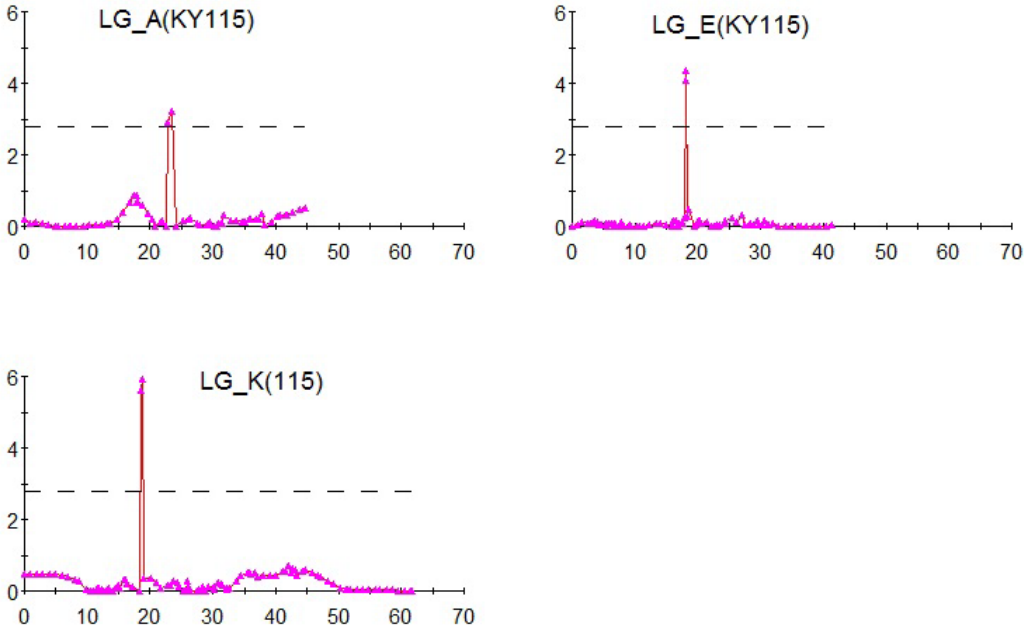


Fig. 2**.** Parental KY115 and AD98 maps composed of 626 and 491 non-redundant SNPs. Red color indicates a set of 244 loci anchored '*in silico*' to Chinese chestnut reference map. Distorted markers (156 loci) were deleted from LG_I (KY115).

A



B

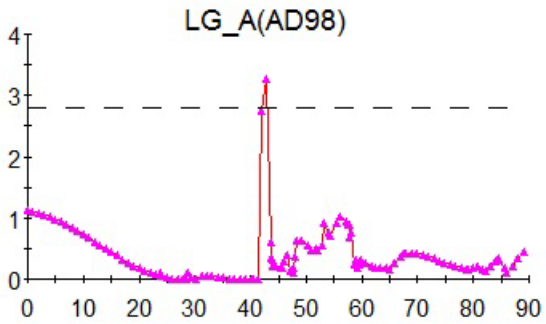


Fig. 3. QTL intervals for resistance to *Pc* on LG_A. LG_E and LG_E identified in female KY115 (A) and male AD98 (B) parents of the HB2-2014 cross with MQM mapping and automatically selected cofactors. Horizontal line represents a LOD significance threshold of 2.8 determined on the basis of 1,000 permutations at a whole genome-wide significance level of $P < 0.05$. The *x*-axis indicates genetic distance (cM) represents genetic distances (cM), whereas the y-axis shows the LOD threshold value. The dashed line represents genome-wide LOD threshold value of 2.8.