

Via Email: sff3@psu.edu

August 4, 2015

Sara Fitzsimmons
The American Chestnut Foundation

RE: Michigan Tech Proposal #1406017 P2, "Identification of different ecotypes and centers of adaptive genetic diversity in American chestnut II," submitted by Dr. Oliver Gailing

Dear Ms. Fitzsimmons:

On behalf of Dr. Gailing, Michigan Technological University is pleased to submit the attached proposal for your review and consideration.

For any questions or concerns of a legal or contractual nature please contact me at (906) 487-2228 or mkhaapap@mtu.edu. For technical questions please contact Dr. Gailing directly at either (906) 487-1615 or ogailing@mtu.edu.

Sincerely,



Marilyn Haapapuro
Associate Director for Industrial Contracting

Progress report: Identification of different ecotypes and centers of adaptive genetic diversity in American chestnut

Principal investigators:

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Outputs

Plant material and marker analyses

A total of nine populations (~32 samples per population) that covered the distribution range of the species (Table 1, Kubisiak and Roberds, 2005) have been characterized at genetically mapped EST-SSRs (Kubiasik et al., 2013). We have tested a total of 25 EST-SSRs that have been developed in *Castanea mollissima*, 17 of them with clear and polymorphic amplifications products were selected for the population analysis. Until now 10 markers have been characterized in all samples and a first data analysis has been performed. We have developed a multiplex PCR touchdown protocol that allowed us to analyze up to four markers in one PCR reaction. The touchdown program in the Biometra Thermocycler Tprofessional (Jena, Germany) was as follows: initial denaturation at 95 °C for 15 min, 10 touchdown cycles of 1min at 94 °C, 1 min at 60 °C (- 1 °C per cycle) and 1 min at 72 °C, followed by 25 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 20 min. The PCR mix consisted of 0.2 µl (5 U/ µl) Hotstar Taq polymerase from Solis Biodyne (Estonia), 1.5 µl 10 X reaction buffer B, 1.5 µl MgCl₂ (25 mM), 0.2 µl (5 pikomol/µl) tailed forward primer, 0.5 µl (5 pikomol/µl) pig-tailed reverse primer (Kubisiak et al. 2013), 1.5 µl M13 primer (5 pikomol/ µl), 1 µl dNTPs (2.5 mM each dNTP), 2µl DNA (ca. 0.6 ng/ µl) and 5 µl H₂O. PCR products were separated on an ABI 3130xl Genetic analyzer (Applied Biosystems) and alleles were called using Genemapper v. 4.0 (Applied Biosystems).

Data analyses

Genetic variation in populations was assessed as the number of alleles per locus (N_a), observed heterozygosity (H_o) and expected heterozygosity (H_e) (Nei, 1973) in the program GeneAEx v.6.41 (Peakall and Smouse, 2006) (Table 2). Genetic differentiation among populations and between population pairs was calculated as F_{ST} . Additionally Nei's unbiased genetic distance (Nei, 1978) between populations was assessed. A Principal Coordinate Analysis was performed to represent genetic distances between populations. Additionally, the program STRUCTURE (Pritchard et al., 2000) was used to determine the number of genetic clusters K and to assign individual samples to genetic clusters following the approach described in Lind and Gailing (2013).

Preliminary results

Genetic variation

Gene diversity or expected heterozygosity (H_e), observed heterozygosity (H_o) and allelic diversity (N_a) were highest in population MNC and lowest in population PCT (Table 2). No latitudinal variation in genetic diversity was observed. However, N_a showed a significant decline from west to east ($R^2 = 0.856$). The northernmost population OCA (Canada, Ontario) showed the second highest values for H_e and N_a , but comparative low values for H_o which might reflect moderate levels of inbreeding.

Genetic differentiation

The eastern populations MA, NYUC and PCT were differentiated from the western populations as revealed by the Principal Coordinate Analysis based on Nei's unbiased genetic distances (Figure 2). PCA 1 explained 62.62% of the variation and was significantly associated with longitude ($R^2 = 0.860$, $p < 0.001$) (Figure 3). Likewise, the STRUCTURE analysis identified two genetic clusters and separated the eastern populations MA, NYUC, PCT from the western populations, while PYC (Pennsylvania) seemed to occupy an intermediate position (Figure 4).

The results suggest a significant east-west differentiation potentially as result of limited gene flow across the Appalachian range. We also identified significant genetic differentiation between populations from one region which might suggest different local adaptations (e.g. between Portland and Massachusetts).

Plans for year 2

Additional 10 to 25 markers will be tested for amplification and polymorphism to obtain a total of ~ 30 markers for the outlier screens. Outlier screens will be performed at all markers in population pairs from contrasting environments. We will search for associations between geographic (longitude, latitude) and environmental variables (e.g. minimum and maximum temperature, precipitation) on the one hand and allele frequencies and genetic variation parameters on the other hand using stepwise regression analysis (Kubisiak and Roberds, 2005) and association mapping approaches.

Budget

A total of \$2,000 is requested for the second year of the program. We will do additional marker tests, outlier screens and the final data analyses. The original proposal including all references is attached.

Table 1 Sample locations

Name	N	Latitude	Longitude
Massachusetts_a2-a40	32	42.22	72.31
Asheville_br2-br35	32	35.46	82.10
Ontario_ca2-ca34	32	43.08	80.30
Portland_CT1-CT33	32	41.35	72.37
Murphy_NC (fc1-jb18)	32	35.05	84.01
Kentucky_pc1-pc40	32	37.50	83.51
New York_uc1-uc29	32	41.44	74.13
Pennsylvania_yc1-yc35	32	39.48	76.59
Maryland_md15-md58)	32	39.37	79.07

Table 2 *Castanea mollissima* primers tested for amplification and polymorphism in *C. dentata*

Marker_name	GenBank_ID	primer1_sequence	primer2_sequence	Motif	BLASTN	LG and position (cM)
CmSI0031	290474526	AGCGCCACTTTTCTTTTCA	GAATCCCAGCCTGACCAATA	AAG	-	LGH (25.3 cM)
CmSI0049	290474533	CCGATGCCGATTTCTACAAC	GCGGCAGACACATAGTTCA	TCTTCA	-	LGB (34.1 cM)
CmSI0051	290474536	CGATCATATCCCATACCCACA	GCGGAGACCACTAAGAGACG	CT		
CmSI0327	290474585	CTCCTCCGCCTCCATTCA	AGTCCTTGCGGATCACTTTG	AG	60S ribosomal protein L27-like, 8e-43	LGK (5.8 cM)
CmSI0383	290474601	CCTCCTCCACCACCAGTTTA	TGGAGGTGGGGACTTGTACT	CTC		
CmSI0391	290474604	TTTGAGTGCTGAGGAGACT	GACTCAAAGTCTCGGCAAA	TAA	-	
CmSI0392	290474605	CCATCGGGAAATGTGGTATT	GCTCATCTGGGAACCACTGT	GTG		LGC (26.4 cM)
CmSI0396	290474606	AACTCCCACCACTCACATCC	TTTCGGACCATCCAGAACTC	CACACC	uncharacterized protein, 1e-4	
CmSI0437	290474621	GGGCTTCTTGAAAAGTAGCA	CCATACGAACACCGAGGACT	TG		LGH (49.4cM)
CmSI0495	290474633	GAAACAACAGGCTCTGCCTC	CTGGGAAAATCCGAACTCAA	GA	probable amino-acid acetyltransferase NAGS2, 9e-78	
CmSI0527	290474668	TACGACCTAAACGACTCGCC	AGGAGGAGAAATCAGCCACA	GTT	-	LGF (14.8 cM)
CmSI0537	290474676	AGAGATGGGTTGGGAAGGTT	GGCCTCTCTGGTTTGTGTGT	AG	-	LGA (41.4 cM)
CmSI0541	290474681	CCAATCCCAAAATCCACTTG	GGACATTTTGGAGCCTGAAA	GA		
CmSI0551	290474692	TAACCAATCACGTTCCACCGA	CGCCACATCTAAAACCCCTA	CGC	-	LGH (26.3 cM)
CmSI0559	290474700	AGGTGGGAGTGAAAGGTGTTG	TATCTCGCTCGCTCCATCTT	AGG	-	LGC (33.3cM)
CmSI0561	290474702	CGTATAGGGTGGAACGGAA	GGACAAGCAAATCACGGAAT	TCG	-	
CmSI0594	290476538	GCCCCAAAGAAAGAGAAAAG	GCATGCCCATACCCATTAAC	GGT		LGH (17.2 cM)
CmSI0600	290474736	TCACACTCACACGCAAAA	TGTTCACGAAATACGCAACG	CT	Uncharacterized locus, 1e-74	
CmSI0603	290474740	ACTCCATGGGAATGATGAGC	TGTGTGTGTGTGTTCTCTGTGA	TC		
CmSI0608	290474745	TTTGATTGGCCCTCCTAGAC	CTGAATCGCCGAACTCATT	AGG	transducin/WD40 repeat-like superfamily protein, 9e-32	LGL (17.8 cM)
CmSI0611	290474746	GCTGACCCTGTCAACAACAA	CAGAACTAGACAAGGATCACAAGA	TC	-	LGK (20.1 cM)
CmSI0632	290474764	TCGGAGTAGTTGGAGCAGTG	TGAGAAAAGGAAAGTGCCTCA	GTT		
CmSI0678	290474795	GGTCAGACCCGCTAGCTCT	ACCCAAAACAAAACCAAAA	TCT	-	LGK (19.3 cM)
CmSI0683	290474802	CACCAGCACCTCACTTCTCC	CCGGAAGATTTAGGTTTAGGG	AGA	-	
CmSI0689	290474809	TCCCAAATGAAATGAAATGAAA	TGAAAATCCCTCCATCATCA	TGAGA		LGD (55.8 cM)

Markers labeled in bold face amplified easy-to-interpret and polymorphic loci and were characterized in all nine populations. BlastN 2.231 results (Zheng et al., 2000) and sequence descriptions are included. Linkage group (LG) position (cM) on the *C. mollissima* linkage map (Kubisiak et al., 2013) is indicated.

Table 3 Genetic variation within populations

Pop		N_a	H_o	H_e	F
ABR	Mean	4.706	0.451	0.468	0.084
	SE	0.527	0.064	0.060	0.067
KPC	Mean	4.706	0.481	0.498	0.050
	SE	0.460	0.061	0.057	0.028
MA	Mean	3.882	0.499	0.489	-0.034
	SE	0.410	0.045	0.044	0.034
MMD	Mean	4.471	0.493	0.475	-0.047
	SE	0.576	0.059	0.057	0.024
MNC	Mean	5.824	0.517	0.531	0.033
	SE	0.637	0.064	0.062	0.043
NYUC	Mean	4.059	0.513	0.489	-0.058
	SE	0.424	0.059	0.057	0.018
OCA	Mean	5.294	0.477	0.517	0.084
	SE	0.435	0.050	0.049	0.042
PCT	Mean	3.706	0.439	0.396	-0.101
	SE	0.400	0.052	0.045	0.029
PYC	Mean	4.471	0.490	0.475	-0.033
	SE	0.394	0.055	0.052	0.030
Total	Mean	4.569	0.484	0.482	-0.002
	SE	0.164	0.019	0.018	0.013

SE: Standard error, N_a: number of alleles per locus, H_o: observed heterozygosity, H_e: expected heterozygosity, F: inbreeding coefficient, $F = (H_e - H_o)/H_e$

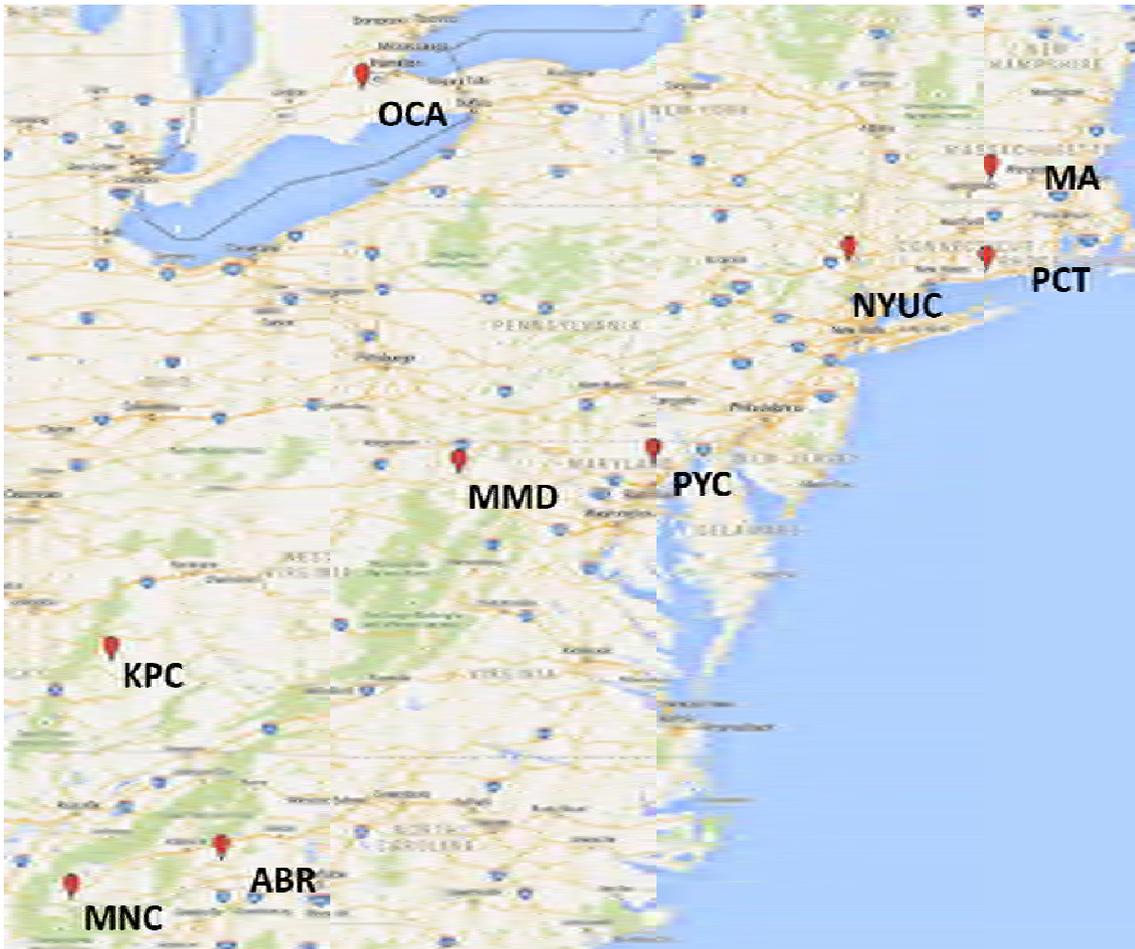


Figure 1. Sample locations

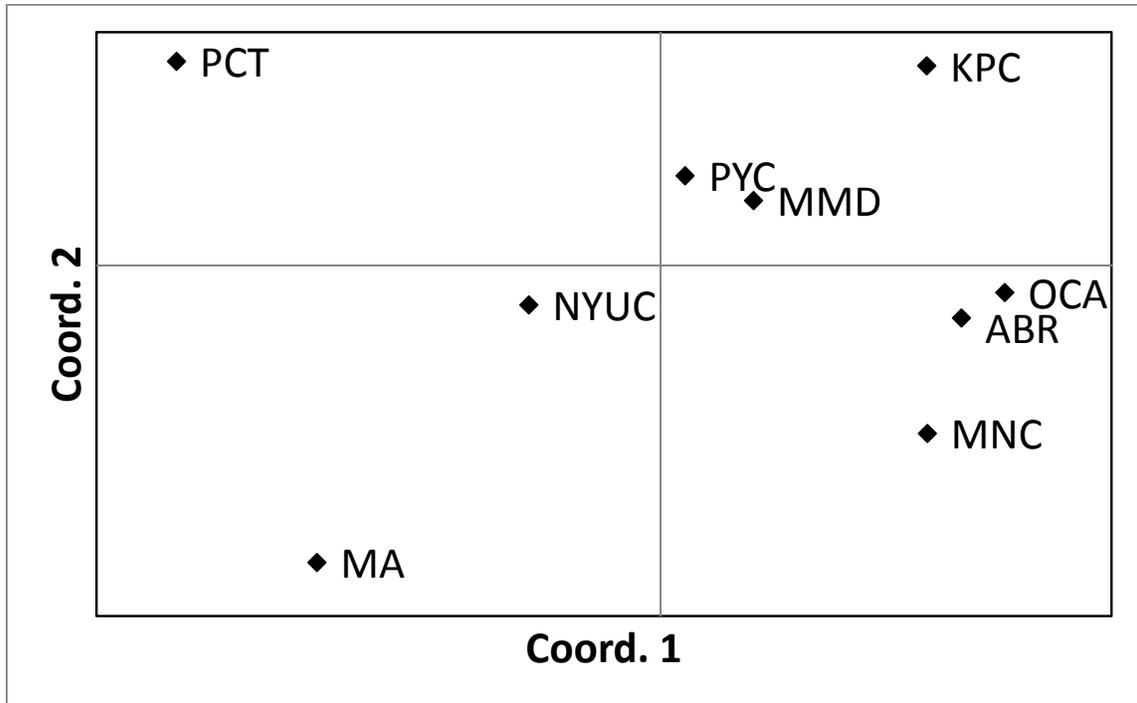


Figure 2. Principal Coordinate Analysis based on Nei's unbiased genetic distance (Nei 1978).

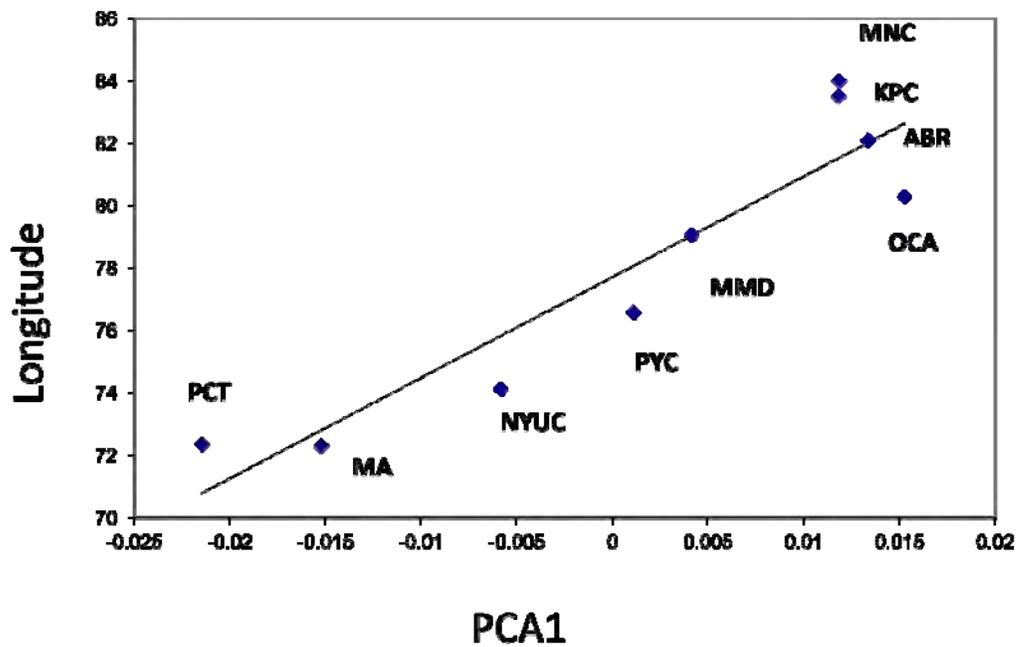


Figure 3. Association between longitude and PCA1.

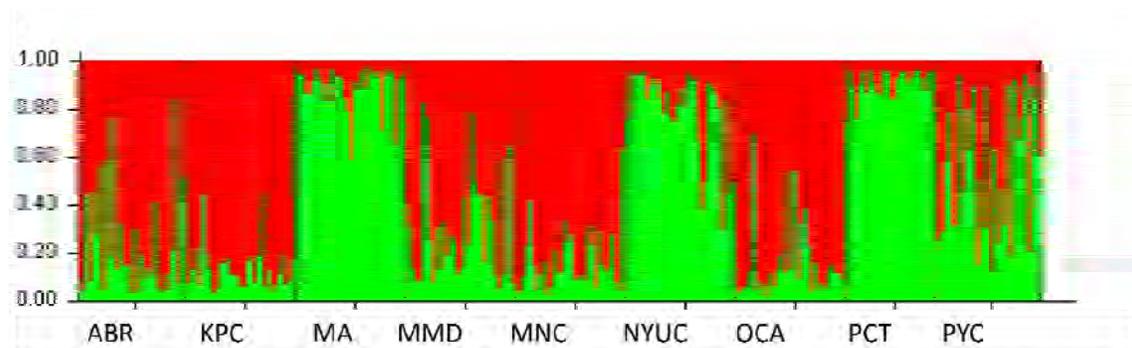


Figure 4. STRUCTURE plots for K = 2.

a. Title: Identification of different ecotypes and centers of adaptive genetic diversity in American chestnut

b. Summary:

We will develop a set of new gene-based DNA markers with potential role in local adaptation to (1) identify centers of genetic diversity in American chestnut, (2) detect gene-based markers under divergent selection in contrasting environments and to (3) associate allele frequency distributions at gene markers with environmental variables. We will map the genes back to the *Castanea mollissima* linkage map (*in silico* mapping) to analyze the distribution of these genes on the linkage map and their potential association with QTL for phenotypic and resistance traits.

c. Principal investigators:

Oliver Gailing, Associate Professor for Ecological Genetics, Michigan Technological University, 1400 Townsend Drive, Houghton 49931, Michigan, ogailing@mtu.edu.

Brian C. McCarthy, Professor of Forest Ecology & Chair, Dept. Env. & Plant Biology, 416 Porter Hall, Ohio University, Athens, OH 45701-2979 USA, mccarthy@ohio.edu

C. Dana Nelson, Project Leader/Research Geneticist, US Forest Service, Southern Institute Forest Genetics, Southern Research Station, Saucier, MS 39574 USA, dananelson@fs.fed.us, phone: 228-832-2747-201

d. Duration of project: 2 years

e. Total amount requested: We requested a total of \$ 6,000. The estimated costs for the marker analyses are \$ 9,500 excluding labor costs. The additional costs will be covered with internal funds.

f. Short and long-term goals of the project: Short-term goals of the project are the identification of (1) center of genetic diversity and (2) of genes under divergent selection in contrasting environments.

The long-term goals are to (1) provide information for the selection of breeding material and reintroduction of hybrid American chestnut and (2) to use a combined outlier screening and QTL mapping approach to identify genes related to biotic and abiotic stress resistance for use in marker-assisted breeding.

g. Narrative

Background

Considerable progress has been made to develop blight resistant chestnuts for restoration purposes using mainly genetic marker-assisted back-cross breeding to incorporate blight resistance from *C. mollissima* into a *C. dentata* genetic background. Restoration programs are more likely to be successful if hybrid American chestnut populations are genetically diverse (James et al., TACF project 2010 - 2013) and locally adapted. For this purpose genetic variation of the American chestnut parents should be captured from many individuals originating from different geographic regions and climatic zones. The conservation of genetic variation in fitness-related traits (adaptive genetic variation) is crucial for the successful restoration of American chestnut since the species' reintroduction is threatened by other biotic (e.g. *Phytophthora cinnamomi*) and abiotic stressors. While growing genomic resources and gene-based markers are becoming available for American chestnut and related species (Barakat et al., 2012; Bodénès et al., 2012; Kubisiak et al., 2013; Nishio et al., 2011), genetic variation at these markers with annotated function (e.g. gene-based microsatellites) has not yet been analyzed in natural populations of American chestnut. In a preliminary study we have already identified and characterized a set of gene-based markers in American chestnut (Table 1).

We hypothesize (1) significant differences in the level of genetic variation for populations from different geographic regions and (2) significant differentiation among regions at some gene-based markers that reflect different local adaptations of the populations (ecotypes) across their distribution range.

These markers that are identified as under divergent selection between populations (outlier loci) from contrasting environments and/or associated with environmental variables across populations will be mapped back to the *Castanea mollissima* linkage map (Kubisiak et al. 2013) to test for a possible co-location with QTL regions. The expected results will be important to identify centers of genetic diversity and to select appropriate breeding material to produce locally adapted material for the reintroduction of American chestnut.

In the future, a combined outlier screening and QTL mapping approach based on nextgen sequencing markers will allow us to test for a co-location of genome-wide outliers with genomic regions that underlie QTL for traits related to biotic and abiotic stress resistance.

Work plan

We propose to characterize genetic variation within and among *C. dentata* populations covering the distribution range of the species using 16 gene-based microsatellite markers with annotated function (Expressed Sequence Tag- Simple Sequence Repeats, EST-SSRs). In this preliminary study we will focus on 10 populations that represent the five US climatic zones within the species' native range. Leaf material has been sampled from about 30 trees per population recording the GPS position of each tree (Kubisiak and Roberds, 2005). In order to select the 16 gene-based microsatellite markers for the

range-wide study, we will screen about 30 markers with annotated function in abiotic and biotic stress tolerance and known location on genetic linkage maps (Kubisiak et al., 2013) in two populations from different climatic zones. We will include orthologous candidate genes that had been identified as being under divergent selection in interfertile red oak species with different adaptations to drought (Lind and Gailing, 2013; Sullivan et al., 2013; Lind-Riehl et al. 2014). A total of 12 of these markers have already been tested in a panel of 8 American and Chinese chestnut individuals and 7 of them amplified polymorphic products in the expected size range (Table 1, Figure 1, unpublished results). Two of the loci, FIR013 and FIR039, were identified as putative outlier loci under strong divergent selection in the North American red oak species and have putative functions in drought stress response and the control of flowering time (Sullivan et al. 2013; Lind-Riehl et al. 2014). EST-SSR markers that display signatures of selection relative to potentially neutral genomic SSRs that had been analyzed in the same sample set (Kubisiak and Roberds, 2005) will be selected for the population analyses. Calculation of genetic variation within and among populations will follow standard procedures. Associations of genetic variation patterns with geographic distance and environmental parameters will also be tested. A detailed description of the methods is given below.

Methods

Marker selection

We have already identified 7 gene-based markers for American chestnut that were originally developed for oak (Durand et al. 2010, Lind-Riehl et al. 2014). Additional EST-SSRs that are genetically mapped in *Castanea mollissima* (Kubisiak et al., 2013) will be selected based on their annotated function in vegetative bud burst and biotic and abiotic stress tolerance, and genomic co-location with QTL for chestnut blight resistance. Our aim is to obtain at least 30 markers for the outlier analysis. Selection of the markers will be based on their reproducibility, absence of null alleles and potential importance in adaptation and disease resistance. Functional annotation will be assigned to microsatellite-containing ESTs using the BLASTx algorithm (Altschul et al., 1997) by comparison to homologous sequences in the NCBI database. Based on the initial outlier screen of the 30 polymorphic markers in two populations from different climate zones (outlier analysis), 16 EST-SSRs will be selected for the population analyses.

Marker analyses

DNA and leaf samples will be provided by Dana Nelson and Tom Kubisiak who performed a range-wide sampling for the assessment of neutral genetic variation patterns at genomic SSR and RAPD markers (Kubisiak and Roberds, 2005). We will be able to combine data sets and compare patterns of genetic variation at candidate gene associated EST-SSR markers with neutral variation (Kubisiak and Roberds, 2005). Samples will be amplified with fluorescent labeled forward primers using a Peltier Thermal Cycler (Geneamp® PCR system 2700, Applied Biosystems) and PCR products will be separated on an ABI PRISM® 3730 Genetic Analyzer (Applied Biosystems). PCR reactions will be

adapted from Kubisiak et al. (2013). Even though species identity was tested with a chloroplast marker that differentiates between American chestnut and the native cogener species chinkapin (*Castanea pumila*) (Kubisiak and Roberds, 2005), the occurrence of interspecific hybrids cannot be excluded. We therefore use the generated marker information to assign individual samples to species and interspecific hybrids in the program STRUCTURE 2.3.4 (Pritchard et al., 2000). For this purpose we will include 20 *C. pumila* reference samples that were identified based on morphology and chloroplast marker information.

Outlier screens

A total of 30 EST-SSRs will be selected for the outlier screen and amplified in two populations from different adjacent climate zones to identify gene loci (outlier loci) that show a higher or lower differentiation between populations than expected under selective neutrality. We will use the program LOSITAN that implements the F_{ST} -based algorithms of FDIST to identify outliers that deviate significantly from a simulated neutral confidence envelope (Antao et al., 2008; Beaumont and Nichols, 1996). Loci with higher differentiation between populations than expected under neutrality are identified as potential outliers under divergent selection. Those falling below the lower bound of the neutral envelope might be under balancing selection. Since the confidence interval converges at extreme values for expected heterozygosity (H_e), candidate genes under balancing selection were not consistently identified in different simulations (Sullivan et al., 2013) while loci under divergent selection were highly reproducible (Sullivan et al., 2013). We will therefore run the simulations at least three times for each pairwise comparison. To identify signatures of selective sweeps we will also run the LnRH test statistic that estimates variability between populations at individual loci instead of population divergence to identify selection (Schlötterer, 2002).

Identification of genetic diversity centers

Genetic variation within and among populations and climatic regions will be calculated for all markers and separately for potentially adaptive (outlier markers) and neutral markers. Specifically the following genetic variation parameters will be calculated: number of alleles per locus (N_a), observed heterozygosity (H_o) and Nei's unbiased gene diversity (H_e) (Nei, 1973). Pairwise genetic differentiation between populations and corresponding significances will be calculated in GenePop4.1 (Raymond and Rousset, 1995). To visualize genetic distances among populations an unweighted pair-group method with arithmetic means (UPGMA) dendrogram (Sneath and Sokal, 1973) will be calculated in Populations 2.0 (Langella, 1999) using 1,000 bootstrap replicates. An Analysis of Molecular Variance (AMOVA, (Excoffier et al., 1992) will be performed in Arlequin 3.5 (Excoffier and Lischer, 2010) in order to assess genetic variation within and among populations and climatic regions. To test for associations between geographic and genetic distances we will perform a Mantel test as implemented in GeneAIEx v.6.41 (Peakall and Smouse, 2006).

Association of allele frequencies with environmental variables

We will search for associations between geographic (longitude, latitude) and environmental variables (e.g. minimum and maximum temperature, precipitation) on the one hand and allele frequencies and genetic variation parameters on the other hand using stepwise regression analysis (Kubisiak and Roberds, 2005) and association mapping approaches. Associations between SSR alleles and environmental variables will be performed using a standard association mapping approach by substituting the phenotype with environmental variables according to Eckert et al. (2010b). Specifically, following Du et al. (2013) associations between SSR marker locus and climatic variables will be analyzed using a mixed linear model accounting for population structure and kinship (Yu et al., 2006) and P-values will be generated for each test using 10,000 permutations of genotypes with respect to environmental values. All analyses will be conducted using TASSEL v. 3.0 (Bradbury et al., 2007). Multiple testing corrections will be performed using the false discovery rate (FDR) method (Storey, 2002; Storey and Tibshirani, 2003). Data on six nuclear microsatellite loci and 19 RAPD loci available for the same populations (Kubisiak and Roberds, 2005) will be used to estimate pair-wise kinship coefficients in the software SPAGEDi (Hardy and Vekemans, 2002) and population structure coefficients in STRUCTURE 2.3.4 (Pritchard et al., 2000).

Climate data (annual minimum and maximum temperatures, monthly temperature, precipitation) will be obtained from the PRISM website (Oregon State University, <http://www.prismclimate.org>) and from an ESRI 30 arc-second grid file available at the WorldClim website (<http://www.worldclim.org/current>) according to Eckert et al. (2010a). Accumulated growing degree-days above 5 °C will be calculated and an aridity index will be calculated according to (Eckert et al., 2010a; Eckert et al., 2010b; Sork et al., 2010).

The location of all markers relative to QTL for phenotypic and disease-resistance traits will be assessed on the *C. mollissima* linkage map to analyze the genomic location of genes with signatures of selection.

h. Timeline:

Activity	Year 1				Year 2			
	1-3	4-6	7-9	10-12	1-3	4-6	7-9	10-12
Outlier screening	x	x						
Range-wide marker analyses			x	x	x	x		
Data analysis and publication		x	x	x	x	x	x	x

j. Budget: Total costs are estimated as \$9,500. A total of \$ 6,000 is requested from the American Chestnut Foundation. Based on our experience with these analyses we estimate \$ 2,000 for the marker development and \$ 7,500 for the population genetic analyses (\$25 per sample x 300 samples, including DNA isolation, PCR, labeled primers and genotyping services). These estimates do not include labor costs. *Requested funds:* Year 1: \$ 4,000. Year 2: \$2,000.

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