Using Genetic Information to Inform Redbay Restoration in Laurel Wilt Epidemic Areas[1]

K.E. Smith,[2,5] M.A. Hughes,[3] C.S. Echt,[2] S.A. Josserand,[2] C.D. Nelson,[2,4] J.M. Davis,[5] and J.A. Smith[5]

Abstract

Laurel wilt disease is incited by the exotic fungus *Raffaelea lauricola* and transmitted by the Asian redbay ambrosia beetle (*Xyleborus glabratus*). The disease has spread from Savannah, Georgia in 2002 across the coastal southeast as far south as the Everglades, and in 2014 was discovered as far west as Texas. Mortality is severe, with locations in Florida reporting more than 90 percent loss of redbays, 7.6 to 10.2 cm (3 to 4 inches) in diameter and greater. Surviving redbays from coastal maritime forest ecosystems have been collected and propagated for the study of disease resistance and ultimately restoration planting. Disease severity of artificially inoculated parental trees and their open pollinated offspring will supply evidence for whether resistance is inherited simply as a dominant versus recessive trait, or as a complex, quantitative trait. These data will be used to identify and guide deployment of resistant (or tolerant) materials in areas where redbay has been decimated by laurel wilt disease. Additionally, in order to confirm parentage and potentially access population structure and diversity, simple sequence repeat (SSR) genotyping is underway in this population. Primer sequences were obtained from the Hardwood Genomics Project public resource (http://www.hardwoodgenomics.org/content/redbay-gssrs) and preliminary results, presented here, appear promising.

Introduction

Laurel wilt disease has spread with alarming speed and severity since its introduction in 2002. This relatively new disease has moved through redbay (*Persea borbonia*) and swampbay (*Persea palustris*) populations across the southeastern United States and is threatening the avocado (*Persea americana*) industry in Florida. The causative fungus, *Raffaelea lauricola,* is vectored by the redbay ambrosia beetle, *Xyleborus glabratus*. There is strong concern for the spread of laurel wilt beyond the range of the favored beetle host, redbay. The fungus can incite disease on a wide range of species within the Lauraceae (Hughes et al. 2015) and *Xyleborus* beetles other than *X. glabratus* (six identified so far) can pick up the deadly fungus by infesting the same tree (Carrillo et al. 2014). The disease poses widespread ecological threats to the large number of *Lauracae* species that contribute to canopies throughout the tropics and subtropics. In addition, these populations can act as reservoirs for the beetle and the fungus, threatening commercial avocado growers in Mexico and California.

Some of the fundamental questions surrounding how redbay, as well as other *Persea* species, can best be restored remain unanswered. Simple sequence repeat (SSR) markers are enormously useful tools for the study of population genetics and mapping. The preliminary data presented here demonstrates that the use of SSR markers in redbay has excellent potential. We tested SSR primers in a population of redbay trees that survived laurel wilt disease outbreaks.

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[2] Southern Institute of Forest Genetics, USDA Forest Service, Southern Research Station, 23332 Success Road, Saucier, MS 39574.
[3] College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, 875 Komohana Street, Hilo, HI 96720.
[4] Forest Health and Education Center, Department of Forestry, University of Kentucky, 208 T.P. Cooper Hall, Lexington, KY 40546.
[5] School of Forest Resources and Conservation, University of Florida, 136 Newins-Ziegler Hall, Gainesville, FL 32603. Corresponding author: smithk@ufl.edu.

Methods

DNA was extracted from freeze-dried leaf tissue using the Qiagen DNeasy Plant Mini kit (69104) or using the Macherey-Nagel NucleSpin 96 Plant II kit. Grinding was done with a SPEX Sample Prep Mini-G genogrinder and steel balls.

The microsatellite loci in this study were amplified using primers flanking tetranucleotide repeats and were designed from Illumina high-throughput sequence data provided by Hardwood Genomics, a National Science Foundation-funded project (http://www.hardwoodgenomics.org/). Primer sequences were modified as follows: M13(-19) universal primer sequence was added to the 5' end of forward primers to avoid the need for fluorescently direct-labeling individual primers (Schuelke 2000) and the sequence GTTTCTT was added to the 5' end of reverse primers to avoid non-template adenylation during PCR (PIG-tailing, Brownstein et al. 1996).

Amplifications of PCR were performed in 10-µl reactions containing 20 ng genomic DNA, 0.04 µM and 0.16 µM of forward and reverse primers, respectively, 0.16 µM FAM labeled M13 primer, 2.64 µM dNTPs, 1X Invitrogen PCR buffer, 2 mM $MgCl_2$, and 0.5 U ''hot start'' Platinum Taq DNA polymerase (Invitrogen, 10966026). The PCRs were completed using the following touchdown protocol: 2 min at 94 ºC; followed by 20 cycles of 30 s at 94 ºC, 30 s at X, and 1 min at 72 ºC where X = 65 ºC in the first cycle decreasing by 0.5 ºC every cycle thereafter; followed by 25 cycles of 30 s at 94 ºC, 30 s at 55 ºC, 1.5 min at 72 ºC; followed by a 15 min extension at 72 ºC. The resulting PCR products were separated on an ABI 3710 Genetic Analyzer (Applied Biosystems, Foster City, California) as recommended by the manufacturer. GeneMapper Software 5 (Applied Biosystems) was used to size the peaks in base pairs and to score alleles, with LIZ600 as an internal size standard.

Results and Discussion

In order to confirm the feasibility of using SSRs as a genetic tool in redbay, we tested 96 primer pairs using seven trees collected from five locations (Cumberland Island, Georgia; Fort Clinch, Florida; Huntington Island, South Carolina; Fort George Island, Florida; Edisto Island, South Carolina). Of the 96 primer pairs, seven failed to amplify and three had weak amplification; 25 were monomorphic in all seven genotypes; 36 could not be scored because of one or more criteria (poor amplification, inconsistent peak heights, hedgehog peaks, peaks with large shoulders, stray peaks, peaks too close together); 35 amplified cleanly and were scored. These 35 primer pairs produced nearly 100 alleles, including a primer pair producing seven alleles in the sample of seven trees. This preliminary result of 35 of 96 (37 percent) loci easily scored, a large number alleles and the large number of redbay SSR primer pairs publicly available (table 1), indicate that it would be relatively efficient to obtain enough loci for fingerprinting or genetic mapping. This seems especially likely considering the genetic map of closely related *P. americana* consists of 25 SSR loci, that are contained in 10 of 12 mapped linkage groups and were obtained by screening only 92 primer pairs polymorphic at the population level (Sharon et al. 1997).

Table 1—Publically available redbay ssr primer pairs, Hardwood Genomics Project

Repeat Type	Available	Tested	PCR positive	Polymorphic/ scored
Dinucleotide	16,010	-	-	-
Trinucleotide	1,886	-	-	-
Tetranucleotide	271	96	92	35

A subset of six primers were selected for further testing based on ease of scoring and allele number. They were scored in 57 additional trees, collected as laurel wilt disease outbreak survivors from six locations (additional location: St. Catherine's Island, Georgia). The original seven trees were tested again and the original genotype data were confirmed. In redbay, there were a total of 56 alleles scored, 17 of which were from a single locus and an average of 9.3 alleles per locus. With this high level of polymorphism, we obtained unique genotypes for all trees in the population. In addition, three of the six

primer pairs were tested in *P. palustris*, *P. podadenia* (Mexican species), *P. americana*, and *Cinnamomum porrectum* (a southeast Asia species) and all amplified scorable alleles in each species.

Because SSRs are codominantly inherited and highly polymorphic, they are a good choice for population genetics studies. In the case of redbay, the choice is even more persuasive given the public availability of thousands of primer sequences. The information provided from comparisons between populations can help to ensure gene conservation goals are met in replanting efforts. Because these primer pairs work well in a variety of species, including swamp bay, the same loci should be useful to inform research in other species threatened by laurel wilt disease. In addition, SSR markers can be easily located in genomic sequence as resources become available in the future.

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