



Diversity level of genomic microsatellites in redbay (*Perseaborbonia* L.) generated by Illumina sequencing

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Abstract

Redbay, *Perseaborbonia* (L.) Spreng., is a common evergreen tree or shrub native to the swamp forests of the Atlantic and Gulf Coastal Plains in the United States. In the past decade, redbay populations have suffered extensive mortality caused by the vascular disease Laurel wilt (*Raffaelealauricola* Eichhoff), which has been spread by the exotic redbay ambrosia beetle (*Xyleborusglabratus* Eichhoff). The disease is threatening the economic, ecologic and aesthetic functions of redbay. Early efforts to preserve genetic diversity of the threatened species, through seed and vegetative propagule collections have occurred without the benefit of guidance from a molecular characterization of existing genetic variation. Molecular markers may prove useful in guiding efficient conservation efforts for this species. Here we surveyed 51 genomic microsatellite (gSSR) markers derived from low-coverage whole genome sequencing of redbay with a panel of 25 unrelated redbay trees from eastern South Carolina. When analyzed in an ABI 3730 Genetic Analyzer, 24 markers demonstrated highly informative scores with a polymorphic information content of at least 0.50 and an estimated null allele frequency ≤ 0.1 . The mean observed and effective number of alleles of these 24 sSSRs were 5.29 and 3.06, respectively. The observed heterozygosity ranged from 0.17 to 1.00 with a mean of 0.65, while the expected heterozygosity ranged from 0.16 to 0.90 with a mean of 0.62. Nei's expected heterozygosity and Shannon's Information index were 0.60 and 1.17, respectively. These results indicate high diversity level in the 24 redbay gSSR markers. This is the first report of genetic variation of redbay DNA markers. The gSSRs exhibiting high levels of polymorphism can be applied in characterization of genetic composition and diversity of seed collection and resistant genotypes in redbay germplasm conservation and breeding programs.

Keywords: *Perseaborbonia*, gSSR, microsatellite, next-generation sequencing

Introduction

Redbay, *Perseaborbonia* (L.) Spreng., (Lauraceae) is an attractive aromatic evergreen tree or shrub indigenous to the southeastern United States. Its geographic distribution ranges to Maryland and Delaware in the north, to eastern Texas in the west, and to the tip of Florida in the south, as well as the Bahama Islands. It grows on hammocks, costal dunes, and along swamp edges in mixed hardwoods and maritime forests [2]. The tree's size and growth habit varies considerably over its range, and can attain

a height of over 60 feet and a trunk diameter of 3 feet. Redbay has limited use as a wood material, though its fine-grained wood is useful for cabinets, veneer, and boat trim. The tree's fruits are consumed by a wide variety of birds, rodents, deer, and bears, and persist late into the year, making it an important winter food source [5]. Dried redbay leaves have been used for generations as the "real" Southern bay leaf for flavoring savory foods, considered essential for gumbo. Native Americans used redbay in ceremonies and medicines [6]. Redbay also serves as

a primary host for larval Palamedes swallowtail butterflies [17].

Today, redbay are being threatened by Laurel wilt, a disease caused by the fungus *Raffaelealauricola* T.C. Harr., Fraedrich & Aghayeva, which is vectored by a nonnative, wood-boring ambrosia beetle (*Xyleborusglabratus* Eichhoff) [9]. Once introduced into a redbay host, the pathogen rapidly causes complete crown wilt and death, often within three weeks. Since the first report of the disease in 2003 on Hilton Head Island, South Carolina, and surrounding areas, thousands of redbays have died in the low country of South Carolina, resulting in loss of 75-80% of Hilton Head's redbays by the end of 2004 [10]. In the past few years, more than 90% mortality rate of large redbays has been reported in some affected sites [9,16,18,25]. Reported a decrease of 91 million redbay stems between the years of 2003 and 2011 in Georgia. In Maryland, redbay has been listed as an endangered species [6]. According to a model reported by [26], Laurel wilt disease is spreading at a rate of 54.8 km/year and infection throughout the entire range of redbay is expected in less than 40 years. The United States Department of Agriculture (USDA) Forest Services have initiated germplasm conservation efforts for redbay through collection and storage of seeds at the USDA Forest Service's National Seed Laboratory in Macon, Georgia. In addition, 80 different clones from lingering redbay in areas with more than 95% mortality have been propagated and maintained in Atlanta GA [7]. However, all these redbay materials have not been characterized.

Informative DNA markers are powerful high resolution tools for evaluation of genetic variation at the molecular level. In particular, microsatellites, or simple sequence repeats (SSRs) markers, have become predominant, because they are co-dominant, easily reproduced and scored, highly polymorphic, abundant through the genome, and have higher information content than isoenzyme and dominant markers [23]. To date, however, no DNA markers have been developed in redbay for characterizing its genetic resources, largely due to lack of redbay sequences. As September 2014, there are only 59 redbay DNA sequences in GenBank. Correspondingly, none of the above germplasm collections nor wild populations have been characterized for levels of genetic diversity using molecular markers. Fortunately, an initial set of genomic resources for 10 non-model hardwood tree species, including redbay [19] was generated via low-coverage whole genome sequencing [12] with an Illumina HiSeq 2000 platform. From the redbay sequencing data that had an estimated genome coverage of 1.49X (based on genome size of two other species in *Persea*: *P. americana* (905Mb) and *P. indica* (1,614Mb), a total of 18,167 potentially amplifiable loci were identified with flanking primers (genomic SSRs, gSSRs). This resource provides the opportunity to develop molecular markers for redbay for the first time.

Methods

We evaluated 51 gSSR makers in an ABI 3730 Genetic Analyzer

with 25 unrelated redbay trees in eastern South Carolina that were available at the time of the study (Figure 1). There were at least 8 km between each tree except for tree #12 and #18. These two trees were 2.4 km apart. Primer design, DNA isolation, and PCR with fluorescent dye (6-FAM, VIC, NED, or PET)-labeled primers were described in [24]. The amplicons were separated in an ABI 3730 Genetic Analyzer. Allele calling was performed using GeneMapper (3.7, Life Technologies, NY). MICRO-CHECKER [21] was employed to check for potential genotyping errors arising from large allele drop-out. Observed and expected heterozygosities and polymorphic information content (PIC) were obtained using Cervus 2.0 [13]. Deviations from Hardy-Weinberg equilibrium and the Shannon's Information index were calculated with POPGENE [22].

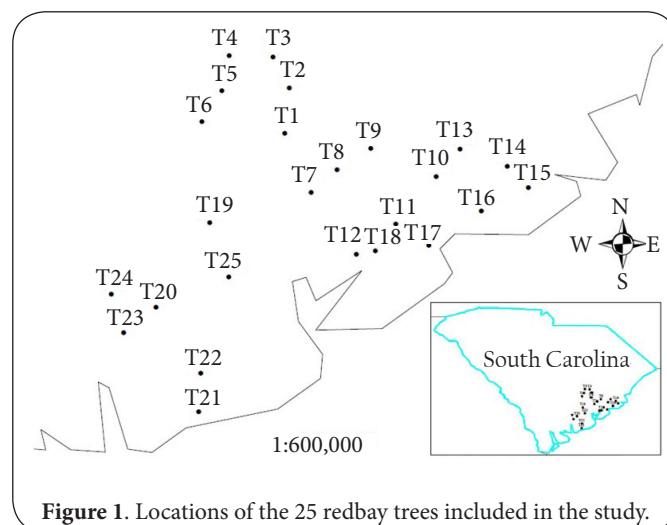


Figure 1. Locations of the 25 redbay trees included in the study.

Results and discussion

We previously tested a subset of 98 markers from the 18,167 potentially amplifiable redbay gSSRs for amplification [19]. All of the 98 markers produced amplicons in at least one of the redbay trees tested, with the exception of marker RBgSSR82. The 51 gSSRs included in this study were randomly selected from the 97 amplifiable markers. Fragment analysis on an ABI 3730XL revealed that only gSSRRBgSSR40 was monomorphic, resulting in a percentage of polymorphic loci of 98%. Amplification with RBgSSR5, 46, 47 and 48 was successful in only one tree, while amplicon sizes of RBgSSR31 and 35 were found to be under 100 bp, smaller than the expected size of 200 bp. No evidence of large allele dropout was found for any of the remaining 44 markers included in further analyses. The type of motif, repeat size, and marker sequences of these markers are listed in **Supplementary Table S1**. Among the 25 redbay trees surveyed with the 44 markers, the number of alleles per locus ranged from 2 to 16 (mean=5.91) (**Table 1**). The observed and expected heterozygosities (H_o and H_e) ranged from 0.04 to 1 and from 0.12 to 0.90, with averages of

Table 1. Statistics of the 44 markers analyzed by cervus.

Locus	k	N	HObs	HExp	PIC
RB2	6	16	0.563	0.673	0.613
RB3	4	21	0.238	0.526	0.475
RB4	5	12	0.833	0.721	0.638
RB6	3	25	0.52	0.58	0.485
RB8	8	24	0.542	0.855	0.819
RB9	5	25	0.64	0.527	0.473
RB10	3	25	0.44	0.496	0.386
RB11	3	25	0.12	0.548	0.43
RB12	3	25	0.04	0.117	0.111
RB13	4	25	0.52	0.543	0.443
RB14	3	25	0.72	0.61	0.528
RB15	7	23	0.174	0.73	0.679
RB16	5	21	0.19	0.663	0.602
RB17	3	25	0.4	0.519	0.414
RB18	3	25	0.32	0.334	0.285
RB19	3	25	0.56	0.626	0.544
RB20	6	25	0.84	0.802	0.753
RB21	5	25	0.72	0.593	0.501
RB22	6	24	0.458	0.556	0.511
RB23	3	21	0.095	0.585	0.501
RB24	2	25	0.96	0.509	0.375
RB25	5	25	0.72	0.687	0.632
RB26	3	24	0.042	0.194	0.178
RB27	3	18	0.167	0.595	0.496
RB29	7	16	0.625	0.788	0.726
RB30	3	24	0.167	0.159	0.148
RB32	5	24	0.417	0.727	0.662
RB33	7	21	0.143	0.823	0.778
RB34	4	25	0.96	0.6	0.502
RB38	6	25	0.8	0.72	0.654
RB39	12	24	0.875	0.891	0.86
RB41	2	24	1	0.511	0.375
RB42	10	25	0.68	0.687	0.651
RB43	9	24	0.5	0.824	0.782
RB44	8	25	0.56	0.795	0.747
RB45	7	24	0.75	0.661	0.601
RB49	15	19	0.684	0.899	0.864
RB50	16	20	0.4	0.899	0.867
RB51	11	22	0.409	0.816	0.773
RB52	11	16	0.5	0.819	0.778
RB53	6	15	0.6	0.768	0.705
RB54	9	16	0.375	0.643	0.605
RB55	5	18	0.167	0.351	0.328
RB56	6	16	0.313	0.688	0.621

K: Number of alleles; N: Number of individuals
 H0: Observed heterozygosity
 HE: Expected heterozygosity
 PIC: Polymorphic information content

0.49 and 0.63, respectively.

The polymorphic information content (PIC) ranged from 0.11 to 0.87, with an average of 0.57. Overall, 29 of the 44 markers demonstrated highly informative scores ($PIC > 0.50$), while 12 were reasonably informative scores ($0.50 > PIC > 0.25$), according to the criteria of [4]. RB12, RB26 and RB30 had a PIC that was less than 0.25. The PIC values obtained indicate that these 41 markers can serve as an important DNA marker resource in redbay population genetic studies. Of these, 17 loci exhibited null alleles however (estimated null allele frequencies range from 0.111 to 0.710 by MICRO-CHECKER), implying that caution should be exercised when using these loci in population genetics studies. When excluding the markers exhibiting null alleles (Table 2), the mean observed and effective number of alleles for the remaining 24gSSR loci were 5.29 and 3.06, respectively. These 24 loci revealed observed heterozygosities ranging from 0.17 to 1.00 with a mean of 0.65, while expected heterozygosities ranged from 0.16 to 0.90 with a mean of 0.62. There was no significant difference between observed and expected heterozygosities ($p = 0.05$, t-Test). Nei's expected heterozygosity and Shannon's Information index were 0.60 and 1.17, respectively. Lastly, a total of 15 loci (RB2, 4, 6, 10, 13, 14, 18, 19, 22, 25, 29, 30, 38, 39, 53) were found to significantly deviate from Hardy-Weinberg equilibrium (Chi-square test, $p > 0.05$) in the 25 trees included in the study (Supplementary Table S2). Thus, these results indicate that the 24 redbaygSSR markers which did not exhibit null alleles could reveal high level of genetic variation among redbay populations.

The study has shown that the approximately 18,000 potentially amplifiable redbay gSSRs generated by low-coverage whole genome sequencing [19] will be a valuable source for developing more informative markers. Compared to EST derived markers, genome sequence-derived SSRs are generally more polymorphic and provide higher resolution [14,20]. Thus, species-specific genomic SSRs are useful for fingerprinting and parentage analysis in closely related lines. Among the species in genus *Persea*, avocado (*Persea americana*) is most intensively studied due to its commercial values as one of the most important fruits in the world. Many DNA markers, SSRs in particular, have been developed and applied in population genetics and mapping of avocado c.f. [1,3]. Some of the avocado EST-SSR markers may be transferable to redbay, as previous studies have shown that EST-SSRs generally demonstrate a high frequency of cross-species transferability [8,11].

From the standpoint of wood utilization, fruit production, or ornamental trade, redbay is not highly important commercially. However, the devastating Laurel wilt, now well established in the southeastern Atlantic Coastal Plain region of the U.S., is causing negative economic, ecological and aesthetic impacts on this native species. This study is the first effort in developing informative molecular markers for redbay germplasm conservation and breeding. This is the first report of genetic variation of redbay DNA markers. The gSSRs characterized in our study,

Table 2. Genetic variation at 24gSSR loci characterized in 25 red bay trees from eastern South Carolina, USA.

25 redbay trees from eastern South Carolina, USA									
Locus	Sample size	Na	Ne	Obs_Hom	Obs_Het	Exp_Hom	Exp_Het	Nei's I	
RB2	32	6	2.88	0.44	0.56	0.33	0.67	0.65	1.33
RB4	24	5	3.24	0.17	0.83	0.28	0.72	0.69	1.33
RB6	50	3	2.32	0.48	0.52	0.42	0.58	0.57	0.93
RB9	50	5	2.07	0.36	0.64	0.47	0.53	0.52	1.01
RB10	50	3	1.94	0.56	0.44	0.50	0.50	0.49	0.74
RB13	50	4	2.14	0.48	0.52	0.46	0.54	0.53	0.89
RB14	50	3	2.49	0.28	0.72	0.39	0.61	0.60	1.00
RB17	50	3	2.04	0.60	0.40	0.48	0.52	0.51	0.80
RB18	50	3	1.49	0.68	0.32	0.67	0.33	0.33	0.57
RB19	50	3	2.59	0.44	0.56	0.37	0.63	0.61	1.02
RB20	50	6	4.68	0.16	0.84	0.20	0.80	0.79	1.63
RB21	50	5	2.39	0.28	0.72	0.41	0.59	0.58	1.04
RB22	48	6	2.19	0.54	0.46	0.44	0.56	0.54	1.13
RB24	50	2	2.00	0.04	0.96	0.49	0.51	0.50	0.69
RB25	50	5	3.06	0.28	0.72	0.31	0.69	0.67	1.33
RB29	32	7	4.23	0.38	0.63	0.21	0.79	0.76	1.60
RB30	48	3	1.18	0.83	0.17	0.84	0.16	0.16	0.33
RB34	50	4	2.43	0.04	0.96	0.40	0.60	0.59	1.01
RB38	50	6	3.40	0.20	0.80	0.28	0.72	0.71	1.37
RB39	48	12	7.84	0.13	0.88	0.11	0.89	0.87	2.24
RB41	48	2	2.00	0.00	1.00	0.49	0.51	0.50	0.69
RB42	50	10	3.06	0.32	0.68	0.31	0.69	0.67	1.59
RB49	38	15	8.02	0.32	0.68	0.10	0.90	0.88	2.37
RB53	30	6	3.88	0.40	0.60	0.23	0.77	0.74	1.53
Mean	46	5.29	3.06	0.35	0.65	0.38	0.62	0.60	1.17
St. Dev.	--	3.16	1.71	0.21	0.21	0.17	0.17	0.16	0.49

Na: Observed number of alleles; Ne: Effective number of alleles [27]; Obs_Hom/Obs_Het: Observed homozygosity/heterozygosity; Exp_Obs/Exp_Het: Expected homozygosity/heterozygosity [28,29] expected heterozygosity; I: Shannon's Information index [30]; St. Dev.: Standard deviation

particularly the 30 markers exhibiting highly informative scores (PIC>0.50), will be useful in studies assessing genetic diversity within and among regions and for conducting paternity analysis in redbay. This study represents the initial and necessary step to future construction of genetic linkage maps, quantitative trait loci mapping for Laurel disease resistance, and enhancing germplasm selection in redbay with molecular markers.

Additional files

[Supplementary Table S1](#)
[Supplementary Table S2](#)

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	CC	YX	TX	MS	GS	OB	SES	JEC	HL
Research concept and design	--	--	--	--	--	--	--	✓	✓
Collection and/or assembly of data	✓	✓	✓	✓	✓	✓	✓	--	--
Data analysis and interpretation	✓	--	--	--	--	--	--	--	✓
Writing the article	✓	--	--	--	--	--	--	--	✓
Critical revision of the article	--	--	--	--	--	--	✓	--	✓
Final approval of article	✓	✓	✓	✓	✓	✓	✓	✓	✓
Statistical analysis	✓	✓	✓	✓	--	--	--	--	✓

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