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White Oak (*Quercus alba* L.) Microsatellite Markers for Genetic Diversity Studies

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Abstract—We screened and validated microsatellite DNA markers (i.e., simple sequence repeats, SSRs) from transcriptome sequences of white oak (*Quercus alba* L.). Of 84 PCR primer pairs previously identified and designed, we found 23 pairs that amplified white oak genomic DNA consistently and were polymorphic among three DNA samples. Subsequently, 16 of these 23 primer pairs were amplified across 225 white oak trees sampled from naturally regenerated stands on the Daniel Boone National Forest in southeastern Kentucky. Population estimates of heterozygosity and total and effective allele numbers averaged 66.4%, 11.3, and 5.5, respectively. These 16 SSR markers proved to be highly polymorphic and provided informative data on the genetic diversity of the white oaks within these stands.

Keywords: Forest genetics; gene diversity; molecular markers; population genetics; silvicultural thinning.

INTRODUCTION

Oaks (*Quercus* spp.) are important species in eastern deciduous forests of North America (Brooke et al. 2019), and white oak (*Quercus alba* L.) specifically has a large effect on key ecosystem processes while also serving as a vital food source for many wildlife species (Fralish 2004, Rogers 1990). White oak ranges from southern Quebec to northern portions of Florida and westward to eastern Kansas (Buchanan and Hart 2012). The species is extensively sourced as lumber for furniture, veneer, paneling, and flooring, but most uniquely harvested as the wood of choice for staves to make bourbon and wine barrels.[1]

Genetic diversity is a key measure of the genetic health of forest stands and the adaptive potential of their constituent species. Genetic markers are useful for characterizing and monitoring genetic diversity

[1] Tirmenstein, D.A. 1991. *Quercus alba*. In: Fire Effects Information System [online]. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Missoula Fire Sciences Laboratory (Producer). https://www.fs.usda.gov/database/feis/plants/tree/quealb/all.html. [Date accessed: 2021 August 31].

within and between areas (e.g., subpopulations, sites, stands, etc.) and across landscapes (Kremer and Hipp 2020). Of the various genetic tools available, microsatellite markers, or simple sequence repeats (SSRs), are especially useful for measuring genetic diversity in forest trees (Echt and Josserand 2018, Nybom et al. 2014).

Microsatellites are short (one to six base motifs), tandemly repeating DNA sequences that are typically abundant and polymorphic in tree genomes making them good candidates for genetic markers (Grover and Sharma 2016). Specific microsatellite markers are assayed by polymerase chain reaction (PCR) amplification from genomic DNA and electrophoresis of the PCR products.

For white oak and other hardwood forest tree species, the Hardwood Genomics Project developed transcriptome sequence data (i.e., DNA sequence data of expressed genes) for several species.[2] From the white oak contig assemblies of these sequences (apparent unique genes or unigenes), 84 SSRs were identified as high-quality microsatellite marker candidates with PCR primer pairs designed (HWG 2008). We evaluated the designed primers for PCR amplification and determination of polymorphism in white oak. Our evaluation found that 23 of the primer pairs consistently amplified interpretable alleles and thus appeared suitable as markers for use in large-scale genetic studies. In this paper, we report the results of this evaluation and a validation experiment scoring 16 of these SSRs over a large sample (225 trees) of white oak.

METHODS AND MATERIALS

Leaf tissue samples were collected from 225 white oak trees using either a hand pruner, pole pruner, or firearm in a preexisting study site on the Daniel Boone National Forest near McKee, KY (Dale 1973, Lhotka 2017). The study site consists of naturally regenerated, white oak-dominated stands representative of medium quality sites (site index 21 m) within the Northern Cumberland Plateau ecological section of southeastern Kentucky. The leaf tissue samples were stored and transported on dry ice in coolers and subsequently stored frozen at the University of Kentucky Forest Health Research and Education Center Lab in Lexington, KY.

Genomic DNA was extracted from the white oak leaf samples using DNeasy Plant Mini Kits (Qiagen, Germantown, MD) following the manufacturer's protocol with a modification to the tissue homogenization step. For homogenization, 100 mg of tissue along with 400 µl DNeasy kit Buffer AP1 and 4 µl RNase A stock solution (100 mg/ml) were placed in a Lysing Matrix A (size 100 × 2 ml) tube (MP Biomedicals, Irvine, CA). The tissue was then homogenized using a Fast Prep machine (MP Biomedicals) and subsequently extracted using the DNeasy protocol. DNA quality and concentration were quantified using a NanoDrop spectrophotometer (Thermo Scientific, Carlsbad, CA) for each of the DNA samples following the manufacturer's guidelines.

For the initial screening, PCR primer pairs for the 84 candidate SSRs (see Introduction) were evaluated by PCR and gel electrophoresis using two DNA samples (M11-46 and M4-142). The forward primers were 5' tailed with the m13 forward (-29) sequence CACGACGTTGTAAAACGAC to facilitate fluorescent labeling (see below), and the reverse primers were 5' tailed with the sequence GTTTCTT to improve amplification fidelity (Echt et al. 2011).

For PCR, we used the following protocol in 25 µl reactions: 4–6 ng of white oak genomic DNA, 0.6 µl of forward primer (0.04 uM), 0.6 µl of reverse primer (0.16 uM), 2.5 µl of 10x TAQ buffer (Platinum TAQ 10x PCR Buffer; Invitrogen, Carlsbad, CA), 0.2 µl of dNTP mixture (1 mM each; 4 mM total all 4 bases), 0.5 µl of TAQ DNA polymerase (Platinum TAQ, 5U/ul; Invitrogen), and sterile nuclease-free water as needed to bring the total volume to 25 µl. The PCR reactions were amplified using the following thermocycling protocol (HWG 2018): 5 min at 95 °C; followed by 35 cycles of 30 sec at 95 °C, 45 sec at 60 °C, and 1 min at 72 °C; and one final extension of 10 min at 72 °C. Following amplification, the PCR fragments were electrophoresed on 2% agarose gels, stained with GelRed (Biotium, Fremont, CA), and visualized and photographed over ultraviolet light.

[2] See the Hardwood Genomics Project knowledgebase and repository website for more information: https://hardwoodgenomics.org/ [Date accessed: 1 July 2022].

After the initial screening, we identified 56 SSR markers that exhibited relatively consistent amplification and a potential for polymorphism between the two DNA samples. These 56 markers were chosen for a second screening using three DNA samples (M11-46, M4-142, and C2-1).

For the secondary screening, we used the following PCR protocol in 6 µl reaction volumes: 4–6 ng of white oak genomic DNA, 0.8 µl of 3-primer mix (0.6 uM forward, 2.4 uM reverse, 2.4 uM labeled m13), 0.6 µl 10x TAQ buffer (KBB buffer B; LCG Biosearch Technologies, Middlesex, UK), 0.4 µl dNTP mixture (1 mM each dNTP), 0.3 µl TAQ DNA polymerase (KlearTaq, 5U/ul; LCG Biosearch Technologies), and sterile nuclease-free water to bring the total volume to 6 µl. The m13 primer was labeled with one of four fluorescent dyes—FAM (blue), VIC (green), NED (yellow), or PET (red) (Life Technologies, Carlsbad, CA).

The PCRs were amplified using the following touchdown protocol: 2 min at 94 °C; followed by 20 cycles of 30 sec at 94 °C, 30 sec at 65 °C minus 0.5 °C/cycle, 1 min at 72 °C; followed by 25 cycles at 55 °C for 30 sec, 72 °C for 1 min 30 sec; followed by a final extension at 72 °C for 15 min. The PCR products were separated by capillary electrophoresis (ABI PRISM 3730xl Genetic Analyzer; Life Technologies) using manufacturer guidelines with an internal size standard (LIZ600; Life Technologies) to facilitate accurate fragment sizing and allele scoring. Fragment sizes, in base pairs, were determined using GeneMapper software version 5.0 (Life Technologies) from the exported capillary data files. A standardized allele scoring and naming protocol was used to call and maintain allele names over capillaries and runs (Deemer and Nelson 2010). From these 56 primer pairs, we found 23 that yielded clear results and polymorphism among the three DNA samples (table 1), and 11 additional pairs that showed promising results but appear to require protocol modifications to be useable (table 1 note). From this secondary screening, we selected 16 SSRs for further validation in a population-level study of 225 trees sampled from the McKee site.

For the population study, we used the same PCR, thermal cycling, and electrophoresis protocols as in the second screening, except that following amplification the PCRs were pooled by marker into four sets (or pools) that were organized to minimize fragment overlap during electrophoresis. Each marker pool contained only one marker per color (FAM, VIC, NED, or PET) so they could be identified separately for scoring with GeneMapper. The allelic data were scored and calibrated as above (detailed in Deemer and Nelson 2010) and analyzed using the add-in GenAlEx 6.5 in Microsoft Excel software (Peakall and Smouse 2006, 2012). For each marker, we calculated number of alleles (Na), effective number of alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), and Hardy-Weinberg Equilibrium (HWE) p-values.

RESULTS AND DISCUSSION

To develop reliable genetic markers for white oak diversity studies, we evaluated 84 SSR primer pairs that were designed using DNA sequence data from expressed genes (transcriptome sequence contigs). Our evaluation proceeded in steps, first using the 84 primer pairs and two DNA samples, then 56 primers pairs and three DNA samples, and finally 16 primer pairs and a population sample of 225 trees including the previously used samples.

Of the 84 SSR primer pairs tested, 56 (67%) showed reasonably consistent PCR amplification and potential polymorphism on 2% agarose gels. Of these 56 pairs, 23 were clearly found to be polymorphic among the three DNA samples that were genotyped in the secondary screening using capillary electrophoresis. The allele calls (labelled as the approximate length of the amplified fragment in base pairs) for the three samples with the 23 polymorphic SSR markers are shown in table 2.

Genetic diversity metrics for each of the 16 markers that were tested over the entire 225 trees from a population sample of white oak are presented in table 3. The number of alleles ranged from 5 to 19 (mean 11.17), and the number of effective alleles ranged from 1.96 to 10.05 (mean 5.35). Observed and expected proportion of heterozygotes varied from 0.51 and 0.49 to 0.82 and 0.90, respectively. The range in missing data across the 16 markers was from 0% to 32% (mean 9%). Across the whole population, the observed and expected number of

Table 1—Twenty-three microsatellite (SSR) marker primer pairs consistently amplifying white oak (*Quercus alba*), with respective repeat motif, number of repeats, DNA sequence of forward and reverse primers, and predicted PCR amplified fragment size in base pairs

Marker name[a]	Repeat motif (alpha minimum)	Number of repeats	Forward primer, 5'-3'	Reverse primer, 5'-3'	Fragment size (bp)	Contig name_SSR
16T*	AAG	10	CACCAGCACCTCACTTCTCC	GGATTTGGGTTTGGGTTGGG	231	WO454_contig2605_v2_ssr246
19T*	ATT	10	GGGCTTGCTTGCATTACTCC	AGATTTGTTCTCTCAATCTCGCC	136	WO454_contig200_v2_ssr66
22T*	AAG	16	CGCACTCTCTCTCAAGGACC	GCTTTCCTTCATGAGCCG	237	WO454_contig9485_v2_ssr208
30T*	GTT	10	TTATCCTGTGGTGCCGTAGC	GCGTTGTCGAACTAGAACCG	116	WO454_contig20836_v2_ssr191
31T*	AAG	8	CTTGCATTGGAAGCAGTCGG	TCATGAAGAACACACACGATGC	195	WO454_contig15547_v2_ssr249
32T*	GGT	7	GATTCGATTTCTTCCGCGGC	CTGGAACTCCACGCTAAAGC	116	WO454_contig11646_v2_ssr392
33T	AGT	9	AGTACTCGAAGCTGGTTGGC	CGAATCCGACTCCGATTTCC	179	WO454_contig15502_v2_ssr159
36T	AGG	8	CCACCATCACTCACTCCACC	CTTCCAGCCTCTATAGCCGC	235	WO454_contig5018_v2_ssr87
39T	CT	9	ATGTGGCTTGTTTAATCGCC	GGAAATTAACTAGTTTGGCCATCCC	122	WO454_contig10533_v2_ssr74
43T*#	CT	8	TGGAGAACGCGAAGATGAGG	CACCGACACTGCTACATTTGC	272	WO454_contig19338_v2_ssr508
44T*	AT	10	TCTGAGAGACGAGGCTGAGC	ACATTACAGCCATTCCCTTTGC	167	WO454_contig352_v2_ssr186
45T*	AG	10	CAGAATCTCCTTCTCCGCC	ACGTAAGGAGAACCGTAATCAGG	236	WO454_contig1356_v2_ssr43
61T*	AG	11	GGAGGGTGGTAGTACATGCG	ACAGCTACACACACAAACCC	226	WO454_contig11887_v2_ssr231
62T*	CT	8	GGAGGCCCTGGTAGAAATGC	AGGTTAACTGGTTATAGCCCACC	158	WO454_contig8805_v2_ssr560
66T*	CT	9	TAGGAACTTCAACGCCACGG	CAAACAGGCCAACTCTAACCC	107	WO454_contig5224_v2_ssr108
68T	AG	8	AAGTGCAAGAGTCCACAGGC	ACTCAGGCATGGGCTACAGC	294	WO454_contig15601_v2_ssr65
70T	CT	9	TGCCTTCAATTCGGATAAGG	CTGGGTTGGTTCTGGAGAGG	310	WO454_sontig8130_v2_ssr77
71T	CT	9	TCTTCATCCACCTTCACCACC	CTTGGAAATGGTGGGCATTGTGC	275	WO454_contig10070_v2_ssr28
73T*	AG	11	TCCTTCACTTGCTGCTGTGG	GTGTGTGTGAGTCTTCGTCG	137	WO454_contig15318_v2_ssr339
75T*#	CT	16	TGGAGAACGCGAAGATGAGG	CACCGACACTGCTACATTTGC	272	WO454_contig19338_v2_ssr406
76T*	AG	8	ACTAAGAGGAGCACCAACGC	TGGCTGTAGAAAGATGGTGTGG	148	WO454_contig17935_v2_ssr194
77T	AG	11	TGGTGATGAGCTAGCTAGTGG	ACAACTGCAACACCAACACC	134	WO454_contig15566_v2_ssr146
82T*	AG	10	TTCAACACCTCAACTTCACG	TCAAACCCGTGACATGACCC	264	WO454_contig4106_v2_ssr151

[a] An asterisk (*) indicates the 16 polymorphic markers that were tested at the population level; a pound sign (#) indicates markers (43T and 75T) that were inadvertently designed from the same contig, providing the same primer pair.

Note: Eleven additional markers (SSR# from bio_data 1963026; ssr97-20T, ssr179-21T, ssr164-25T, ssr32-26T, ssr82-27T, ssr164-28T, ssr169-29T, ssr156-35T, ssr248-38T, ssr72-42T, ssr216-63T) may prove useful with appropriate protocol modifications (see text).

heterozygotes differed significantly ($p<0.05$) for 8 of the 16 markers. The reduction in the number of observed heterozygotes relative to expected was also apparent in the HWE p-values, with the same eight markers testing significant ($p<0.05$) for departure from equilibrium. The four markers with the most missing data (from 18% to 32%) may benefit from modifications in PCR conditions to improve their performance. In addition, the 7 markers of the 23 that were not run across the whole population (markers in table 2 pool5 and not pooled) sample should be further tested for their potential usefulness in population studies.

The validated set of 16 SSR markers (15 unique loci, see table 1) should prove useful for examining the population genetic structure of white oak across the Cumberland Plateau region and likely throughout the species range. Studies examining SSR markers for white oak are not common and usually have only a few primer pairs tested. For example, a study on population differentiation among three species of white oaks in northeastern Illinois (Craft and Ashley 2006) used five SSR markers developed for swamp white oak (*Q. bicolor*) and bur oak (*Q. macrocarpa*). The number of SSR markers that are now available for white oak has been expanded with the development and validation of these 16 SSRs in *Q. alba*. Additionally, the seven primer pairs that passed our initial and secondary screening but were not evaluated in the population study could perhaps yield additional polymorphic markers with further optimization of primer design or PCR amplification conditions.

Table 2—Alleles (approximate fragment length in base pairs, bp) scored by marker and DNA sample (C2-1, M11-46, and M4-142) in the secondary screen

Allele pool[a]	DNA samples, grouped by marker											
Pool1	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**
Marker	19T	19T	19T	31T	31T	31T	45T	45T	45T	66T	66T	66T
Allele 1		159	168	207	210	210	268	264	268	130	130	130
Allele 2		162		210			272				132	134
Pool2	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**
Marker	22T	22T	22T	32T	32T	32T	76T	76T	76T	82T	82T	82T
Allele 1	249	246	237	140	133	133	170	178	174	284	281	283
Allele 2		255	249	143	140		176	200	182	286	283	286
Pool3	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**
Marker	30T	30T	30T	43T	43T	43T	61T	61T	61T	62T	62T	62T
Allele 1	139	139	143	290	284	292	247	252	257	183	181	181
Allele 2		219		296	296	298	252	259	261			183
Pool4	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**
Marker	16T	16T	16T	44T	44T	44T	73T	73T	73T	75T	75T	75T
Allele 1	252	252	249	193	193		166	148	152	290	284	292
Allele 2			255	200	195		168	166	158	296	296	298
Pool5	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**
Marker	33T	33T	33T	36T	36T	36T	68T	68T	68T	77T	77T	77T
Allele 1	198	198	198	249	249	249	315	315	323	157	149	157
Allele 2	201				252		325	328		165	157	163
Not pooled	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**	**C2-1**	**M11-46**	**M4-142**			
Marker	39T	39T	39T	70T	70T	70T	71T	71T	71T			
Allele1	119		123	312	307			273	273			
Allele2			125	314				275	277			

[a] Each pool (pool1-pool5) is a set of four markers grouped together for capillary electrophoresis.

CONCLUSION

Our data suggest that the 16 SSR markers evaluated and validated in this study will be highly useful for white oak conservation, management, and tree improvement programs. The genetic diversity information provided by these markers will help to inform practitioners on the genetic health of their white oak populations as well as to ensure the species long-term ecological and economic sustainability.

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Table 3—Population experiment results by marker

Marker name	N	Na	Ne	Ho	He	HWE p-value[a]
16T	217	6	1.955	0.51	0.488	0.654
19T	221	11	4.696	0.52	0.787	*< 0.001
22T	217	11	4.285	0.82	0.767	0.279
30T	219	8	2.567	0.59	0.61	*< 0.001
31T	219	5	3.009	0.65	0.668	0.981
32T	221	5	2.163	0.46	0.538	*< 0.001
43T	201	15	9.18	0.73	0.891	*< 0.001
44T	179	10	5.842	0.6	0.829	*< 0.001
45T	196	14	7.399	0.58	0.865	*< 0.001
61T	176	14	8.017	0.7	0.875	*< 0.001
62T	184	9	3.191	0.64	0.687	*< 0.001
66T	222	11	3.597	0.74	0.722	0.881
73T	153	13	5.97	0.79	0.833	*< 0.001
76T	222	19	10.05	0.78	0.9	*< 0.001
82T	225	11	5.404	0.75	0.815	*0.002

[a] An asterisk indicates a statistically significant (> 0.05) p-value.

N=number of trees, sample size; Na=number of alleles observed; Ne=effective number of alleles; Ho=observed heterozygosity; He=expected heterozygosity; HWE=Hardy-Weinberg Equilibrium.

Note: 43T and 75T represent the same locus (see table 1 note) and provided nearly identical population data; however, only 43T data are presented here as it was easier to score due to 75T being somewhat overloaded in the capillary electrophresis runs.

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To file a program discrimination complaint, a complainant should complete a Form AD-3027, *USDA Program Discrimination Complaint Form*, which can be obtained online at https://www.ocio.usda.gov/document/ad-3027, from any USDA office, by calling (866) 632-9992, or by writing a letter addressed to USDA. The letter must contain the complainant's name, address, telephone number, and a written description of the alleged discriminatory action in sufficient detail to inform the Assistant Secretary for Civil Rights (ASCR) about the nature and date of an alleged civil rights violation. The completed AD-3027 form or letter must be submitted to USDA by:

(1) Mail: U.S. Department of Agriculture
Office of the Assistant Secretary for Civil Rights
1400 Independence Avenue, SW
Washington, D.C. 20250-9410; or

(2) Fax: (833) 256-1665 or (202) 690-7442; or

(3) Email: program.intake@usda.gov.

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