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Primer Note

Identification and characterization of microsatellite loci in the tuliptree, *Liriodendron tulipifera* (Magnoliaceae)[1]

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- *Premise of the study:* Twenty-three polymorphic microsatellite loci (simple sequence repeats) were identified and characterized for *Liriodendron tulipifera* (Magnoliaceae), a species native to eastern North America, to investigate its genetic diversity, population structure, and mating system.
- *Methods and Results:* Using Illumina HiSeq paired-end reads from genomic DNA, searches for repeat motifs identified approximately 280,000 potentially amplifiable loci. Of 77 loci tested, 51 amplified consistently. When genotyped using 30 to 52 total adult trees from three old-growth populations in Maryland, Virginia, and New Jersey, USA, 23 loci were polymorphic. These loci exhibited four to 13 alleles, and observed and expected heterozygosities ranged from 0.233 to 0.865 and 0.272 to 0.876, respectively.
- *Conclusions:* The microsatellite marker loci presented here will be valuable in population genetic studies of *L. tulipifera* because they do not suffer from ascertainment bias and show high polymorphism.

Key words: *Liriodendron tulipifera*; Magnoliaceae; microsatellites; next-generation sequencing; population genetic studies; simple sequence repeats (SSRs); tuliptree.

Liriodendron tulipifera L., commonly known as tuliptree, tulip poplar, or yellow poplar, is a pioneer tree in the family Magnoliaceae native to eastern North America. It has a wide geographic distribution in the southeastern and mid-Atlantic United States and occurs in diverse habitats. To facilitate population genetic analyses of effective population size and population structure, we developed genomic microsatellite (simple sequence repeat [SSR]) markers without the potential limitations of previously reported SSRs. *Liriodendron tulipifera* SSRs have been developed from expressed sequence tags (ESTs; Xu et al., 2006, 2010; Yang et al., 2012; Zhang et al., 2015) located in or near functional genes, and consequently, they are more likely to be affected by natural selection (Ellis and Burke, 2007). *Liriodendron chinense* (Hemsl.) Sarg. genomic (noncoding, nontranscribed) microsatellite loci have been cross-amplified in *L. tulipifera* (Yao et al., 2008). Cross-species amplification of microsatellite loci might result in ascertainment bias, where polymorphism is reduced when loci are transferred to related species (Ellegren et al., 1995). Preliminary tests of loci from Yao et al. (2008) carried out with 10 *L. tulipifera* individuals showed low polymorphism (results not shown). Nonneutral evolution or ascertainment bias can potentially impact the estimation of population genetic parameters. Therefore, we identified and characterized polymorphic genomic microsatellite loci in *L. tulipifera* using Illumina next-generation sequencing and a bioinformatics pipeline.

METHODS AND RESULTS

***Microsatellite development*—**Total DNA from leaves of one *L. tulipifera* individual collected on the main campus of Georgetown University in Washington, D.C., USA, was extracted using the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). A genomic DNA library for Illumina paired-end sequencing was prepared from 4 µg of DNA following the PCR-free library prep kit from Illumina (Illumina, San Diego, California, USA). DNA was sheared to 550 bp and sequenced as 150 bp paired-end reads on an Illumina HiSeq 2500 at the Biocomplexity Institute of Virginia Tech (Blacksburg, Virginia, USA). We used PAL_FINDER_v0.02.04 (Castoe et al., 2012) to extract reads containing perfect microsatellites (uninterrupted and identical repeats). The reads were imported to PAL_FINDER and analyzed in two different ways: (1) as Illumina paired-end reads filtered to include ≥12 tri-, ≥10 tetra-, ≥8 penta-, and ≥6 hexanucleotide repeats, and (2) as FASTQ sequence files converted to FASTA format, treated as 454 single-end reads, and filtered to include ≥15 di-, ≥10 tri-, ≥8 tetra-, ≥6 penta-, and ≥4 hexanucleotide repeats. One potential advantage of using both methods is the development of loci with a broader range of amplification fragment sizes. In both cases, we identified microsatellite loci with flanking sequences suitable for PCR primer design or potentially amplifiable loci (PALs). Raw reads were deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA; BioProject no. PRJNA331147, BioSample no. SAMN05417503). Summaries of reads containing microsatellite repeats and PALs (with primer sequences) detected using both methods are available in Appendices S1 and S2.

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We selected a set of 77 PALs to empirically assess amplification using three individuals. We amplified each locus in 25-µL PCR reactions (1× OneTaq Standard reaction buffer, 160 µM dNTPs, 0.2 µM forward primer, 0.2 µM reverse primer, 0.625 units OneTaq DNA polymerase [New England BioLabs, Ipswich, Massachusetts, USA], 1 µL template DNA [concentration was not determined], and ddH_20 to 25 µL). Thermocycling conditions were 94°C (30 s); followed by 30 cycles of denaturation at 94°C (30 s), annealing at 50–61°C (30 s, Table 1), and extension at 68°C (30 s); and a final extension of 68°C (5 min). Fifty-one primer pairs yielded products of the expected size without nonspecific amplification and were then tested for polymorphism in seven individuals, by visualizing PCR products on 3% agarose gels. Of these 51 loci, 23 were polymorphic and used to genotype 30 to 52 total individuals collected from three old-growth locations in the native range of *L. tulipifera* (Appendix 1). Because *L. chinense*, the other single species in *Liriodendron* L., has a restricted geographic distribution in

Table 1. Characteristics of 23 polymorphic genomic microsatellite loci isolated from *Liriodendron tulipifera*.

Locus	Primer sequences (5′–3′)	Repeat motif	Observed amplicon size (bp)	T_a (°C)	Fluorescent labeling method[a]	Identifier[b]
Lt006	F: GTGGTAACGCATGAGATGGC R: TGAGCTTTCCATCAGGTGAGC	$(AAG)_{12}$	416–437	58	M13-labeled	KX869968
Lt011	F: GCATGGACATGGTGTAACCC R: TTCCATGTGTGCCCTACTGC	$(AAT)_{12}$	223–250	58	6-FAM[1]	KX869967
Lt014	F: CTCACATGAACAACAAGGAAACC R: CTGGGATCTGCACTGATTGG	$(AAT)_{16}$	108–126	58	M13-labeled	HWI-1KL163:137:H7DJKA DXX:1:2116:14211:16050 1:N:0:CTTGTA
Lt023	F: CGTGGCCAGCATCTTGTAGC R: CAAGAACAACGAGGCAAACAGC	$(AAC)_{16}$	113–140	58	M13-labeled	HWI-1KL163:137:H7DJKAD XX:1:2214:4579:5389 1:N:0:CTTGTC
Lt025	F: AGTTGGGAATTGGGACAAGG R: TTCAGTGCTCCAGTTTTCACG	$(ATC)_{13}$	105–120	57	M13-labeled	HWI-1KL163:137:H7DJKAD XX:1:2114:7509:71461 1:N:0:CTTGTA
Lt032	F: GCTCCTACAAACATCAAAAGC R: CAAAACCCATTTCGTGTTCC	$(AAG)_{13}$	95–122	50	M13-labeled	HWI-1KL163:137:H7DJKAD XX:1:2109:2516:54908 1:N:0:CTTGTA
Lt035	F: CACAGAGCTTGGTGCTTTACG R: AAGTCCATGTTCCACTCATTCG	$(AAT)_{16}$	89–119	56	NED[1]	HWI-1KL163:137:H7DJKA DXX:1:1115:14496:36717 1:N:0:CTTGTA
Lt036	F: TTGAAGTTTGAATCCCCATCC R: TGATTGGGCCATGTTAATCG	$(ATC)_{13}$	107–143	50	VIC[3]	HWI-1KL163:137:H7DJKA DXX:1:1109:19109:85000 1:N:0:CTTGTA
Lt043	F: TCATCTTCCTTTGGTTTGCC R: TGGGGATTTGACAGAGAACG	$(AAG)_{12}$	119–149	50	6-FAM[3]	HWI-1KL163:137:H7DJKAD XX:1:2103:5825:78158 1:N:0:CTTGTA
Lt052	F: TGGTCCCGAGATTGTTCACC R: TCTTACCCACCACAACCATCG	$(AAC)_{14}$	94–115	57	M13-labeled	HWI-1KL163:137:H7DJKAD XX:1:2106:8132:35499 1:N:0:CTTGTA
Lt054	F: CCTCGTAGTGTTGATCGTTGC R: TCAACCTTCCACCAGTGTCC	$(AAT)_{16}$	88–121	57	NED[2]	HWI-1KL163:137:H7DJKA DXX:1:1214:10945:60086 1:N:0:CTTGTA
Lt059	F: CTGCCCCTTCAAATTCTTGG R: AATTGCGTGAAGCTCAGACC	$(AGG)_{12}$	93–111	55	NED[3]	HWI-1KL163:137:H7DJKA DXX:1:2102:16887:46302 1:N:0:CTTGTA
Lt060	F: CCTACTCTTCCGGAGTTTCG R: GGGATGGGGTGGAATATAAGC	$(AAT)_{16}$	126–150	57	M13-labeled	HWI-1KL163:137:H7DJKAD XX:1:1215:8150:67169 1:N:0:CTTGTA
Lt061	F: TTGGCGGATGATTGAGAGC R: TTAATGCCGTGGGTTCTGC	$(AAG)_{14}$	122–146	55	PET[3]	HWI-1KL163:137:H7DJKA DXX:1:2205:15636:87476 1:N:0:CTTGTA
Lt064	F: AAGGATGACTTTCACTGAGG R: CATTGGGACTTTATTTCTCTCC	$(AAT)_{13}$	120–156	53	6-FAM[2]	HWI-1KL163:137:H7DJKA DXX:1:1203:17908:71099 1:N:0:CTTGTA
Lt066	F: TCTGGCCCTTGATACTGTGG R: CCCACTTGGGTGTTTCAGG	$(ATC)_{12}$	134–152	57	M13-labeled	HWI-1KL163:137:H7DJKAD XX:1:1109:1769:97275 1:N:0:CTTGTA
Lt068	F: AAACTCCCTAACAAGGGTCTCC R: ACCACAACACAGAAACAATGGG	$(AAT)_{16}$	89–131	55	VIC[2]	HWI-1KL163:137:H7DJKAD XX:1:2211:4001:84575 1:N:0:CTTGTA
Lt070	F: TTCTCCGCCATCGTCTTACC R: CTAATGAACGGTCGGGATGG	$(ATC)_{12}$	112–133	57	6-FAM[1]	HWI-1KL163:137:H7DJKA DXX:1:2211:16362:43312 1:N:0:CTTGTA
Lt075	F: CATCGCCATTGTTTTCTCTGC R: TAACTGCCTGCGTATCATCC	$(AAT)_{13}$	119–134	55	M13-labeled	HWI-1KL163:137:H7DJKAD XX:1:1201:4554:96377 1:N:0:CTTGTA
Lt077	F: TATCCAAACGGCCCTTAACC R: GATCACAAAACTCCCACATGC	$(AAT)_{14}$	84–105	55	HEX[1]	HWI-1KL163:137:H7DJKAD XX:1:1214:2279:95306 1:N:0:CTTGTA
Lt079	F: GGGAGACTCGGCTTTAATCGCC R: GAGTGGAGTAGCGGGACAGG	$(ATC)_{12}$	76–94	61	PET[2]	HWI-1KL163:137:H7DJKA DXX:1:2215:19866:60074 1:N:0:CTTGTA
Lt080	F: GGCCTGAATTCCTTTGTTCCC R: CCCTCAATGTACACGCTTGC	$(AAT)_{12}$	130–151	57	M13-labeled	HWI-1KL163:137:H7DJKAD XX:1:1107:4232:34637 1:N:0:CTTGTA
Lt081	F: ATGGATTCCGGCAAGTCTCC R: TGAGGAAGAGAACAAACAGGGG	$(AAT)_{17}$	96–123	57	M13-labeled	HWI-1KL163:137:H7DJKA DXX:1:2115:20835:65097 1:N:0:CTTGTA

Note: T_a = annealing temperature.

[a] PCR multiplex sets are indicated as 1, 2, or 3.

[b] Numbers are either GenBank accession numbers or Illumina sequence identifiers associated with NCBI's Short Read Archive (BioProject no. PRJNA331147, BioSample no. SAMN05417503).

TABLE 2. Genetic properties by individual and pooled sampled locations of 23 polymorphic microsatellite markers developed in *Liriodendron tulipifera*.[a]

	Montpelier (n = 20)				Frog Canyon (n = 20)				Saddler's Woods (n = 12)				Pooled locations (n = 52)						
Locus	N	A	H_e	H_o[b]	N	A	H_e	H_o[b]	N	A	H_e	H_o[b]	N	A	H_e	H_o[b]	PIC	F	$Null_{IIM}$
Lt006	10	6	0.789	0.800	10	2	0.100	0.100	10	3	0.689	0.700	30	6	0.637	0.533	0.591	0.166	0.074
Lt011	20	6	0.726	0.600	19	7	0.834	0.789	12	5	0.779	0.833	51	9	0.789	0.725	0.749	0.081	0.039
Lt014	10	6	0.768	0.600	10	5	0.800	0.800	10	6	0.816	0.700	30	7	0.821	0.700	0.782	0.149	0.078
Lt023	10	5	0.505	0.500	10	5	0.616	0.600	10	6	0.779	0.800	30	6	0.767	0.633	0.715	0.177	0.075
Lt025	10	4	0.658	0.900	10	2	0.268	0.300	10	3	0.426	0.300	30	4	0.462	0.500	0.420	–0.085	0.056
Lt032	10	5	0.700	0.700	10	5	0.711	0.900	10	5	0.779	0.700	30	8	0.809	0.767	0.773	0.053	0.036
Lt035	19	7	0.750	0.263***	12	6	0.815	0.167***	11	4	0.571	0.273*	42	10	0.826	0.238***	0.793	0.714	0.402†
Lt036	20	8	0.856	0.850	20	8	0.856	0.750	12	6	0.812	0.917	52	9	0.850	0.827	0.822	0.027	0.017
Lt043	20	6	0.805	0.750	20	5	0.703	0.300***	12	7	0.862	0.667**	52	7	0.807	0.558***	0.771	0.311	0.098†
Lt052	9	4	0.699	0.667	10	6	0.684	0.700	10	5	0.805	0.900	29	7	0.782	0.759	0.744	0.030	0.036
Lt054	19	7	0.764	0.579	20	7	0.782	0.700	10	6	0.826	0.100***	49	11	0.806	0.531***	0.773	0.344	0.171†
Lt059	20	5	0.583	0.650	20	4	0.674	0.500*	12	5	0.717	0.917	52	6	0.694	0.654	0.646	0.059	0.030
Lt060	10	5	0.774	0.600	10	5	0.695	0.500	10	7	0.789	0.700	30	9	0.775	0.600	0.733	0.228	0.101
Lt061	20	4	0.458	0.450	20	3	0.555	0.050***	12	3	0.301	0.333	52	6	0.492	0.269***	0.432	0.455	0.130†
Lt064	20	5	0.750	0.600	17	7	0.693	0.176***	12	5	0.725	0.417	49	10	0.825	0.408***	0.791	0.508	0.238†
Lt066	10	4	0.695	0.600	10	4	0.574	0.600	10	2	0.521	0.500	30	4	0.598	0.567	0.519	0.053	0.064
Lt068	20	12	0.837	0.900	20	10	0.873	0.850	12	10	0.783	0.833	52	13	0.876	0.865	0.855	0.013	0.014
Lt070	20	3	0.578	0.450	19	5	0.629	0.632	12	3	0.663	0.667	51	5	0.632	0.569	0.557	0.102	0.059
Lt075	10	2	0.395	0.500	10	2	0.268	0.100	10	2	0.100	0.100	30	4	0.272	0.233	0.254	0.145	0.168
Lt077	20	4	0.742	0.700	19	6	0.725	0.842	12	6	0.641	0.750	51	7	0.765	0.765	0.717	0.000	0.033
Lt079	20	4	0.391	0.400	20	3	0.188	0.200	12	3	0.420	0.417	52	4	0.320	0.327	0.297	–0.021	0.025
Lt080	10	3	0.542	0.200*	10	5	0.774	0.200**	10	4	0.616	0.300*	30	6	0.654	0.233***	0.587	0.647	0.438†
Lt081	10	6	0.811	0.600	10	5	0.774	0.700	10	6	0.763	0.800	30	7	0.809	0.700	0.766	0.137	0.065

Note: A = number of alleles; F = fixation index; H_e = expected heterozygosity under random mating; H_o = observed heterozygosity; n = number of individuals sampled; N = number of individuals genotyped; $Null_{IIM}$ = estimate of null allele frequency given the individual inbreeding model (IIM); PIC = polymorphism information content.

[a] See Appendix 1 for locality and voucher information.

[b] Significant deviation from Hardy–Weinberg expected genotype frequencies: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

† 95% highest posterior density interval does not include zero.

China and Vietnam, we were not able to test for cross-species amplification. As cross-amplification of genomic SSRs has limited success in plants (Merritt et al., 2015) and success declines as genetic divergence increases (Barbará et al., 2007), we did not test for cross-amplification in other Magnoliaceae.

For fragment analyses, PCR products were fluorescently labeled either using primers tailed with a 5′ M13(–21) sequence following Schuelke (2000) or using primers with a 5′ fluorophore and amplified in multiplex (Table 1). In the tailed primer labeling method, two PCR reactions were carried out using the same reverse primer. The first PCR used an M13(–21)-tailed locus-specific forward primer, while the second used a universal fluorescently labeled M13(–21) as a forward primer. The products of the first PCR were purified using StrataPrep PCR Purification Kit (Agilent Technologies, Santa Clara, California, USA) and then used as the template for the second PCR. Fluorescent products were electrophoresed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA), and amplicon sizes were estimated with either orange or red DNA size standard (MCLAB, San Francisco, California, USA) and GeneMapper software 3.7 (Applied Biosystems) using the Local Southern sizing algorithm.

***Microsatellite data analysis*—**Genotypes appeared diploid, displaying at most two alleles per locus per individual. Data were analyzed by sampled location and as a pooled population (Table 2). For each locus, number of alleles, observed heterozygosity (H_o), expected heterozygosity under random mating (H_e), and polymorphism information content ($\mathrm{PIC} = 1 - (\Sigma_{i=1}^{n} p_i^2) - \Sigma_{i=1}^{n-1}\Sigma_{j=i+1}^{n} 2p_i^2 p_j^2$; Botstein et al., 1980) for the pooled population were estimated using CERVUS 3.0.3 (Kalinowski et al., 2007). We used GENEPOP 4.2 (Rousset, 2008) to test deviation from Hardy–Weinberg expected heterozygote frequency (HWE) using default values for Markov chain parameters, and to estimate the fixation index ($F = [H_e - H_o]/H_e$; Hamilton, 2009) for the pooled population.

Population genetic parameters are listed for each sampled location showing loci exhibited two to 12 alleles, with almost all alleles common to each location (Table 2). Lack of population differentiation (F_{ST} = 0.077 estimated using GENEPOP) justified pooling genotypes from the three locations. In the pooled population, observed and expected heterozygosities ranged from 0.233 to 0.865 and 0.272 to 0.876, respectively. Six loci showed significant deviations from HWE (Table 2) with deficits of heterozygotes that could be attributed to numerous causes. One hypothesis is nonrandom mating, which we tested using INEST (Chybicki and Burczyk, 2009) with the individual inbreeding model (IIM) run for 50,000 burn-in and 500,000 total cycles. The estimated average coancestry coefficient over all loci (*f*) was 0.041, with a 95% highest posterior density interval of [0.000, 0.085], indicative of an outcrossing species. Another hypothesis for the deficit of heterozygosity is the presence of null alleles. Frequencies of null alleles estimated with INEST using IIM are listed in Table 2. The six loci with significant deficits of heterozygotes also showed evidence of null allele frequencies greater than zero.

When comparing models including null alleles (*n*), mating among relatives (*f*), and genotyping failures (*b*) using INEST, the full model (*nfb*) was found to best fit the data by the lowest deviance information criterion value (DIC = 5905.837), showing that all three parameters contributed to observed genotype frequencies. The *nb* model, without mating among relatives, exhibited the closest DIC value (5916.991), but the difference was greater than 10, indicating stronger support for the *nfb* model (Igor J. Chybicki, personal communication).

CONCLUSIONS

The 23 microsatellite markers developed here do not suffer from ascertainment bias and show high levels of polymorphism. Taken together with thousands of PALs, this study provides useful resources for population genetic studies of *L. tulipifera*.

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Appendix 1. Locality and voucher information for the *Liriodendron tulipifera* samples used in this study.

Location	County, State	Latitude	Longitude	Voucher no.[a]	*n*
Frog Canyon, Smithsonian Environmental Research Center	Anne Arundel, Maryland	38.884284	–76.552695	MARY1021991	20
James Madison's Montpelier	Orange, Virginia	38.226667	–78.179444	MARY1021990	20
Saddler's Woods	Camden, New Jersey	39.900722	–75.057750	MARY1021989	12

Note: *n* = number of individuals sampled.

[a]A voucher sample for each location was deposited in the Norton-Brown Herbarium (MARY), University of Maryland, College Park, Maryland, USA.