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Source: Applications in Plant Sciences, 1(7)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1200538

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Applications in Plant Sciences 2013 1(7): 1200538

PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF MICROSATELLITE MARKERS FOR *SHOREA PLATYCLADOS* (DIPTEROCARPACEAE)[1]

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- *Premise of the study:* Microsatellite markers were isolated and characterized in *Shorea platyclados* (Dipterocarpaceae) for DNA profiling and genetic diversity assessment of this tropical timber species.
- *Methods and Results:* Fifteen polymorphic microsatellite loci were developed and characterized in *S. platyclados* using a genomic library enriched for dinucleotide (CT) repeats. The primers amplified dinucleotide repeats with 3–14 alleles per locus across four natural populations. The observed and expected heterozygosities ranged from 0.292 to 1.000 and from 0.301 to 0.894, respectively. No significant deviation from Hardy–Weinberg equilibrium was detected in the 15 loci. Four loci pairs displayed linkage disequilibrium.
- *Conclusions:* These highly polymorphic markers are adequate for DNA profiling and studies of population genetics in *S. platyclados*.

Key words: DNA marker; hill dipterocarp; microsatellite; *Shorea platyclados.*

Shorea platyclados Sloot. ex Foxw. is a member of the Dipterocarpaceae, which comprises 500 species across the tropical region (Ashton, 1982). This species can be found in hilly areas of Sumatra, Peninsular Malaysia, and Borneo (Symington, 2004). In Peninsular Malaysia, it has been recorded from an altitude of 300 to 1200 m on ridges, valleys, and hillsides. The timber is traded as Dark Red Meranti in the international market for furniture making, high class interior finishing, flooring, and extravagant doors. With the rising demand in markets for legally harvested timber, many countries are imposing regulations to make sure that the imported timber and timber products are harvested in a sustainable way. The European Union (EU) timber regulations (No. 995/2010) prohibit the placing of illegally harvested timber on the EU market. The EU is negotiating with timber-producing countries to adopt the Forest Law Enforcement, Governance and Trade (FLEGT) license on their verified legal timber.

Therefore, there is a need for a timber tracking system to trace and verify the source of timber until the end-product is received by the consumer. The existing timber tracking systems use paper-based documentation for the identification of timber origin throughout the chain of custody. However, these systems are vulnerable to falsification or tampering, hence increasing the possiblity of illegal timber harvesting. Alternatively, timber tracking based on DNA markers (Tnah et al., 2010) that uses characteristics inherent to the timber can be applied to complement the current control mechanisms. Among the candidate DNA markers are microsatellites with characteristics of codominant inheritance, high degree of polymorphism, and reproducibility (Weising et al., 2005). Here, we characterized 15 microsatellite loci for *S. platyclados* that will be useful for DNA profiling in timber tracking systems and genetic diversity studies.

METHODS AND RESULTS

Total genomic DNA of *S. platyclados* was extracted from leaf tissues using a modified cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). A microsatellite-enriched library was constructed via the magnetic bead hybridization selection approach using 5′-biotinylated $(CT)_{15}$ (Kijas et al., 1994; Lee et al., 2004). Plasmid DNA of positive clones was amplified using the TempliPhi DNA Sequencing Template Amplification Kit (GE Healthcare, Piscataway, New Jersey, USA), and a total of 384 clones were sequenced using BigDye Terminator Sequencing Kit version 3.1 (Applied Biosystems, Foster City, California, USA) on an ABI 3130xl Genetic Analyzer (Applied Biosystems). The results showed 270 unique sequences, indicating 29.7% redundancy. Of the nonredundant sequences, a total of 184 unique microsatellite-containing sequences were identified, of which 93% comprised dinucleotide repeats. A total of 60 primer pairs were successfully designed from sequences that possessed adequate flanking regions, using OLIGO 6.67 software (Molecular Biology Insights, Cascade, Colorado, USA). The primer pairs were designed to be 19–25 nucleotides in length, based on the criteria of lower internal stability at the 3′-end sequence, GC content, and the lack of dimer and hairpin structures.

The initial screening of these primer pairs was done using four unrelated individuals of *S. platyclados*. The PCR amplifications were conducted in 10-μL reaction mixtures, consisting of approximately 10 ng of template DNA, 1× GoTaq Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 μM of each primer, 0.2 mM of each dNTP, and 0.5 U of GoTaq Flexi DNA polymerase (Promega Corporation, Madison, Wisconsin, USA). Each reaction mixture was subjected to amplification using a GeneAmp PCR System 9700 (Applied Biosystems) for an initial denaturing step of 3 min at 94°C; 35 cycles of 94°C, 50–55°C, and 72°C each

[1]Manuscript received 2 October 2012; revision accepted 13 December 2012.

The authors thank J. Ghazali, M. Yahya, P. Ramli, B. Yasri, D. Mariam, T. Sharifah, C. S. Suryani, and H. M. Nurul for their assistance in the field and laboratory. The Terengganu, Perak, Kelantan, and Pahang state forest departments are acknowledged for granting permission to collect samples from the forest. This project was supported by the Ministry of Agriculture and Agro-Based Industry, Malaysia, under the Science Fund (05-03-10-SF1035).

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doi:10.3732/apps.1200538

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TABLE 1. Characteristics of 15 microsatellite loci of *Shorea platyclados*.

Locus	Primer sequences (5′–3′)	Fluorescent dye	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
Spl529	F: GGTCGTTTCATGCTCCCACTTG	6-FAM	$(CT)_{16}$	84–112	50	JX668688
	R: CACTCTGCCTTTGGGAAGTGTTAG					
Spl537	F: ACTTCGACGTGAAATCTTAACATA	6-FAM	$(AG)_8$	150–175	50	JX668689
	R: CGTCCTCTACGTCTTCCTCTTC					
Spl599	F: ATGAACTGATGTTAGATTTACTGT	6-FAM	$(CA)_{10}$	219–240	50	JX668690
	R: GCGTCAGTACCTTAAAAATAGAA					
Spl600	F: TTCTGATTGCCATTTAGTTGTG	6-FAM	$(CA)_{10}$	128–146	50	JX668691
	R: GATCAAATTACTCAAGCATGTG					
Spl629	F: ATTTCTAGCCAAGTAGGTGAGAGT	6-FAM	$(AC)_{11}$	210–226	55	JX668692
	R: TGGGGTAGTATTAGATGAAGTAG					
Spl667	F: CCATTAAAGCATCAAAGTATTG	6-FAM	$(TG)_{12}$	148–184	55	JX668693
	R: TATGAAGAAACTGGAAGGTATTAC					
Spl676	F: CCAGCCCACTATCAACTACCAGAA	6-FAM	$(TC)_{10}$	130–165	50	JX668694
	R: GGCTCCACTTCCGAAAATTGAATC					
Spl690	F: TTGGGGTTTGTTCATGCAACG	6-FAM	$(CT)_{16}$	122–150	55	JX668695
	R: TATTGTTCATTGGGTGTTTCAGAA					
Spl763	F: AGGCAAGCCCTTATATAGTTAGGA	HEX	$(CT)_{16}$	170–196	50	JX668696
	R: GCTGACAGAAGAGTGCAAATATG					
Spl764	F: CATCCCACATCTCTGTATTTGATT	HEX	$(TC)_{14}$	149–178	55	JX668697
	R: ATTTATGCGTGCAGAAACAAACAT					
Spl834	F: CGTCCTCTACGTCTTCCTCT	HEX	$(CT)_{14}$	185–211	55	JX668698
	R: GCAGCCAAAACTTGCAGAAA					
Spl845	F: GGATTAGGTCTTCCTTCTATTTAC	HEX	$(TC)_{15}$	135–175	55	JX668699
	R: CTAATATGAAATGGGATTTTAATG					
Spl855	F: GACTCTGTCCCTCACCAACGACT	HEX	$(TC)_{17}$	196–222	55	JX668700
	R: AAAGCCAATTACAGCACCAAACAC					
Spl858	F: TCGCATGAAATAGATTAGCTGTT	HEX	$(TC)_{14}$	147–182	55	JX668701
	R: ATAGCGGCATCCTCACATGAAT					
Spl863	F: ATATTGTTCATTGGGTGTTTCAGA	HEX	$(AG)_{16}$	109–151	55	JX668702
	R: ACTTGGGGTTTGTTCATGCAAC					

Note: T_a = annealing temperature.

at 30 s; and a 30-min extension at 72°C. The PCR products were electrophoresed on 2% agarose gels. Thirty-six primer pairs successfully amplified products with the expected fragment size and were selected for fluorescent labeling at the 5′-end with either 6-FAM or HEX at the forward primers. The 36 labeled primer pairs were further assessed using four natural populations from Peninsular Malaysia, each consisting of 24 samples. Representative voucher specimens of *S. platyclados* were deposited at the Genetics Laboratory of the Forest Research Institute Malaysia (A1753 from Tembat Forest Reserve [5°11′45.8″N, 102°35′47.8″E]; A1771 from Belum Forest Reserve [5°36′41.8″N, 101°39′20.1″E]; A1680 from Sungai Betis Forest Reserve [4°44′58.0″N, 101°26′42.4″E]; and A1440 from Awana Forest Reserve [3°23′30.4″N, 101°47′20.4″E]).

For genotyping, PCR products were subjected to fragment analysis using an ABI 3130xl Genetic Analyzer (Applied Biosystems) with ROX 400 (Applied Biosystems) as the internal size standard. Allele sizes were assigned against the internal size standard and individuals were genotyped using GeneMapper version 4.0 software (Applied Biosystems). Fifteen loci showed reliable amplification, and allelic polymorphisms could be scored consistently. Table 1 summarizes the characteristics of these microsatellites. Genotypic data were analyzed using CERVUS version 3.0.3 (Kalinowski et al., 2007). From the four populations, the number of alleles ranged from three to 14 (Table 2). The observed and expected heterozygosities ranged from 0.292 (Spl529, Spl690, and Spl863) to 1.000 (Spl676) and from 0.301 (Spl529, Spl690, and Spl863) to 0.894 (Spl676), respectively. Exact tests for

TABLE 2. Genetic variability estimated for the 15 microsatellite loci in *Shorea platyclados* across four forest reserves.

	Tembat Forest Reserve (n = 24)					Belum Forest Reserve (n = 24)					Sungai Betis Forest Reserve (n = 24)					Awana Forest Reserve (n = 24)				
Locus	A	H_o	H_e	HWE[a]	PIC	A	H_o	H_e	HWE[a]	PIC	A	H_o	H_e	HWE[a]	PIC	A	H_o	H_e	HWE[a]	PIC
Spl529	5	0.792	0.668	0.464	0.588	6	0.458	0.571	0.029	0.490	4	0.542	0.471	1.000	0.423	4	0.292	0.301	0.495	0.281
Spl537	9	0.833	0.819	0.939	0.781	7	0.792	0.832	0.793	0.789	10	0.833	0.882	0.519	0.850	7	0.833	0.818	0.920	0.776
Spl599	6	0.875	0.833	0.792	0.789	5	0.708	0.617	0.074	0.551	6	0.708	0.666	0.901	0.601	7	0.875	0.823	0.305	0.777
Spl600	3	0.542	0.643	0.584	0.556	5	0.625	0.579	0.043	0.502	4	0.667	0.592	0.550	0.523	4	0.625	0.679	0.867	0.596
Spl629	5	0.542	0.475	1.000	0.432	7	0.583	0.499	0.274	0.452	6	0.375	0.511	0.051	0.474	4	0.750	0.588	0.287	0.530
Spl667	9	0.810	0.847	0.781	0.806	11	0.958	0.889	0.901	0.858	9	0.909	0.833	0.884	0.794	9	0.833	0.847	0.713	0.808
Spl676	11	0.792	0.879	0.168	0.846	14	1.000	0.894	0.497	0.864	11	0.875	0.855	0.832	0.821	10	0.958	0.858	0.557	0.821
Spl690	5	0.792	0.668	0.466	0.588	6	0.375	0.544	0.007	0.473	4	0.542	0.471	1.000	0.423	4	0.292	0.301	0.498	0.281
Spl763	3	0.667	0.595	0.898	0.495	5	0.625	0.543	0.078	0.470	4	0.583	0.637	0.748	0.564	5	0.750	0.648	0.921	0.573
Spl764	6	0.458	0.635	0.045	0.570	6	0.583	0.667	0.621	0.601	9	0.750	0.795	0.525	0.756	6	0.833	0.681	0.269	0.621
Spl834	10	0.833	0.821	0.903	0.783	8	0.792	0.841	0.533	0.800	9	0.792	0.848	0.393	0.811	6	0.750	0.705	0.782	0.658
Spl845	9	0.833	0.820	0.748	0.776	8	0.583	0.671	0.412	0.599	6	0.708	0.775	0.588	0.719	7	0.917	0.818	0.532	0.774
Spl855	6	0.583	0.562	0.475	0.523	6	0.542	0.664	0.007	0.615	5	0.583	0.509	0.908	0.470	6	0.500	0.598	0.090	0.547
Spl858	9	0.875	0.791	0.962	0.754	9	0.917	0.859	0.014	0.822	10	0.958	0.865	0.625	0.830	7	0.750	0.775	0.667	0.719
Spl863	5	0.792	0.668	0.464	0.588	6	0.458	0.571	0.027	0.490	4	0.542	0.471	1.000	0.423	4	0.292	0.301	0.500	0.281

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; n = number of individuals; PIC = polymorphism information content.

[a]No significant deviation from HWE was detected after conservative Bonferroni correction ($P < 0.003$).

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Hardy–Weinberg expectations were conducted using Genetic Data Analysis (GDA) version 1.0 (Lewis and Zaykin, 2001). After conservative Bonferroni correction, no significant deviation from Hardy–Weinberg equilibrium was detected in all of the 15 loci ($P < 0.003$; Table 2). The 15 loci exhibited relatively high polymorphism information content, ranging from 0.281 to 0.864, with a mean value of 0.630. There was linkage disequilibrium ($P < 0.0005$) between the loci pairs of Spl529/Spl690, Spl529/Spl863, Spl537/Spl834, and Spl690/Spl863.

CONCLUSIONS

The 15 microsatellite markers characterized here for *S. platyclados* will serve as a useful tool to establish a DNA profiling database to monitor and verify the legality of the timber. The availability of these DNA markers provides an additional option for the development of a timber tracking system to accommodate the increasingly stringent laws and regulations in consumer countries. The markers can also be used in genetic diversity studies to formulate conservation strategies for dipterocarps in hilly areas, in particular *S. platyclados*.

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