Development of 12 microsatellite markers in *Euptelea polyandra* by a random tailed genome-walking method using Phi29 DNA polymerase

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Abstract Twelve microsatellite (simple sequence repeat, SSR) markers were isolated from *Euptelea polyandra*, a Japanese endemic deciduous tree species, based on a random tailed genome-walking method using Phi29 DNA polymerase. There were 2 to 11 alleles per locus, and the observed and expected heterozygosities ranged from 0.05 to 0.90 and from 0.233 to 0.883, respectively.

Keywords Microsatellite (SSR) · *Euptelea polyandra* · Random tailed genome-walking · Phi29 DNA polymerase

Euptelea polyandra Sieb. et Zucc. (Eupteleaceae) is a Japanese endemic riparian tree species. For analysis of its population genetics, we first isolated microsatellite (simple sequence repeat, SSR) markers from this species based on a random tailed genome-walking method using Phi29 DNA polymerase (Reddy et al. 2008).

DNA was extracted from leaves of *E. polyandra* using the CTAB method (Zhou et al. 1999). Approximately 12.4 ng of the genomic DNA was mixed with 1- μ l 10 $\times$ reaction buffer, 0.05 mM of each dNTP, and 1 pmol of a random tailed primer Ad1, Ad2, Ad3 or Ad4 (Table 1) in a tube.

After DNA was heat-denatured at 94°C for 5 min and annealed to the primer at 30°C for 5 min, 5 units of Phi29 DNA polymerase (Epicentre Biotechnologies, USA) was added as the final volume of the reaction mixture became 10- μ l. The mixture was incubated at 30°C for 1.5 h for multiple primer extension, and then at 65°C for 10 min for reaction termination. The products were refined with a molecular filter (SUPRECTM PCR, TaKaRa, Japan), resuspended in 20- μ l of TE buffer, and used in the following genome-walking.

The first-step walking was carried out by PCR with 0.4 μ M of a tail primer Ad-P1 (Table 1), and 0.4 μ M of an SSR primer ((AC)₁₀, (CT)₁₀ or (AG)₆(AC)₅) in a 10- μ l reaction solution using a BioTaqTM PCR kit (Bioline, USA), amplifying 3' regions flanking to the SSRs in the genome. The PCR products were integrated into plasmid Novagen pT7 Blue T-Vector (Novagen) using a TaKaRa ligation kit (version 2.1, TaKaRa). The plasmids were transformed into competent *Escherichia coli* (JM 109, TaKaRa), according to the manufacturer's instructions. The cloned fragments were amplified from the plasmid DNA of positive clones using the M13 forward and reverse primers, sequenced using Thermo Sequenase pre-mixed cycle sequencing kits (GE Healthcare, UK), and analyzed using an SQ-5500E DNA sequencer (Hitachi Electronics). Two site-specific primers were designed from the sequence between the tail and SSR sequences. The primers designed from farther and nearer regions to SSR sequence were designated as SP1 and SP2, respectively. For the DNA fragment containing a compound SSR sequence at one end, only one site-specific primer (SP) was designed from the sequence flanking it. The primer pair comprising the site-specific primer (SP) and the compound SSR primer (AG)₆(AC)₃ was used as a compound SSR marker (Wu et al. 2008).

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Table 1 The nucleotide sequences of primers based on Reddy et al. (2008)

	Primer name	Primer sequence (5' → 3')
Random tailed primer	Ad1	GTGAGCGCGCGTAATACGACTCACTATAGGGNNNNATGC
	Ad2	GTGAGCGCGCGTAATACGACTCACTATAGGGNNNNGATC
	Ad3	GTGAGCGCGCGTAATACGACTCACTATAGGGNNNNNTAGC
	Ad4	GTGAGCGCGCGTAATACGACTCACTATAGGGNNNNCTAG
Tail primer	Ad-P1	GTGAGCGCGCGTAATACGA
	Ad-P2	GTAATACGACTCACTATAGGG

The second-step walking contained a nested PCR using the above PCR kit. After the primary PCR with the tail primer Ad-P1 and a site-specific primer SP1, nested PCR was carried out using 1 µl of 100-fold diluted primary PCR products as template with a primer pair of another tail primer, Ad-P2 (Table 1), which was designed from 3'-flanking sequence of Ad-P1, and another site-specific primer SP2. Cloning and sequencing of the PCR fragments were carried out as mentioned above. A locus-specific primer (SP3) was newly designed between Ad-P2 and the SSR. The primer pair SP1/SP3 or SP2/SP3 was used as an SSR marker.

To characterize isolated SSR markers, leaves of *E. polyandra* were collected from 20 individuals distributed in the reverse side of Yotagiri River and Otagiri River in Nagano-Prefecture. The leaves were dried and preserved at room temperature by packing with silica gel in plastic bags until used for DNA extraction. DNA was extracted from dried leaves by the CTAB method. The extracted DNA pellet was resuspended in 200 µl of TE buffer and stored at −20°C. To amplify the SSR markers composed of a locus specific primer pair (Ep01–Ep11), an economic tail method for the amplification of fluorescent-labeled PCR fragments (Schuelke 2000) was adopted. The PCR amplification of SSR markers was carried out using the BioTaq™ PCR kit in a 5-µl reaction mixture containing 0.4 µM of the forward primer (SP1 or SP2), 0.04 µM reverse primer with a tail of M13f and 0.4 µM of M13f primer of which one tenth (0.04 µM) was 5'-labeled with Texas Red (Greiner Bio-One). PCR amplification of compound SSR marker (Ep12) contained 0.4 µM of the forward primer (SP) and 0.4 µM of the compound SSR primer (AG)₆(AC)₃ of which one tenth (0.04 µM) was 5'-labeled with Texas Red. After PCR amplification, DNA samples were denatured by heating at 94°C for 5 min and separated using the DNA Sequencer on a 6% Long Ranger sequencing gel (FMC Bio-Products). The allele sizes were estimated using FraglyS version 3 (Hitachi Electronics Engineering). The observed and expected heterozygosity and the linkage disequilibrium at

each SSR locus were calculated using ARLEQUIN version 2.0 software (Schneider et al. 2000).

In total, twelve polymorphic, codominant SSR markers, including a compound one, were isolated successfully (Table 2). There were two to 11 alleles per locus, and the observed and expected heterozygosities ranged from 0.05 to 0.90 and from 0.233 to 0.883, respectively. With the exception of Ep01, 03, 05, 06 and 09, the observed heterozygosities of all other loci were consistent with expected ones after Bonferroni correction ($P < 0.05$, $n = 20$ individuals). Locus Ep03 showed significant linkage disequilibrium with locus Ep05 ($P < 0.05$). The random tailed genome-walking method using Phi29 DNA polymerase was shown to be effective to develop SSR or compound SSR markers. The method requires no large amounts of sample DNA because the sample DNA is directly amplified by Phi29 DNA polymerase.

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Table 2 Primer pairs for the amplification of 12 polymorphic microsatellite regions in *Euptelea polyandra* and some characteristics of the microsatellite markers

Locus	Primer sequence (5'–3')	T_a (°C)	Repeat	Observed allele size range (bp)	Number of alleles	H_O	H_E	P value	GenBank accession number
Ep01	F: ATTCCTTAGGGAGGCCTATTG R: tail-GGCTAACCTTAGCTCAAATC	54	(TG) ₁₅	142–156	4	0.05	0.701	0.000	AB492053
Ep02	F: GACTCAACCCAAATGTGGC R: tail-GTGGGAACTCAATAATTTAGC	54	(GT) ₉	154–164	5	0.65	0.699	0.478	AB492054
Ep03	F: CTTGGGTTTCTGTCTTACA R: tail-AAACCCATTAAATAGTACAAA	52	(TA) ₄ (TG) ₈	125–153	8	0.35	0.805	0.000	AB492055
Ep04	F: GAGAAAGCACTTGTAAAAGAG R: tail-CTTTACATCCTCGATTATCAC	52	(TA) ₁₃	136–146	6	0.45	0.718	0.002	AB492056
Ep05	F: GTTTCTTTAATGGAGGAGTTG R: tail-GAAACCATTTGATCTTCATCTGC	54	(AG) ₂₀	159–273	11	0.25	0.847	0.000	AB492057
Ep06	F: GTCTTTTCATCTTTTGTGCAGTC R: tail-ACTGTACCTTGAGAAAGCATC	54	(AG) ₁₀ GG	92–116	9	0.20	0.846	0.000	AB492058
Ep07	F: AGATCAGCGCATTAAGACTGA R: tail-AGTGCATCTGCATCTGTATG	54	(AG) ₃	131–170	8	0.90	0.826	0.999	AB492059
Ep08	F: ATGAAGACCCATCGGTGG R: tail-TCCTTCTTCCATGTTAGCGT	54	(CT) ₁₆	137–197	8	0.75	0.883	0.301	AB492060
Ep09	F: CTTGGTTTTGCAAAACCCCGAG R: tail-AATGAAACGAAAGCCCGGATT	54	(CA) ₆ ...(AG) ₄ ...(AG) ₆ (TG) ₈ ...(AG) ₅ (TG) ₄	184–194	6	0.35	0.771	0.000	AB492061
Ep10	F: AGATGGCCTGAATCCATTGA R: tail-GCTTGGCCGGAAAATATCTGA	54	(GT) ₇	129–135	2	0.10	0.233	0.152	AB492062
Ep11	F: TTGTTGTCTCTCAATCCTGAC R: tail-AGTGGATGTTAGCCGGTTTAT	54	(AT) ₆ GT	172–174	2	0.40	0.422	1.000	AB492063
Ep12	F: CCAAGCTTTGAAAGCTCGGAT R: (AG) ₆ (AC) ₃	54	(AG) ₁₈ (TG) ₉ (TC) _n	58–70	8	0.70	0.796	0.428	AB492064

With the exception of Ep01, 03, 05, 06 and 09, the observed heterozygosity of all other loci was consistent with that expected under Hardy–Weinberg equilibrium after Bonferroni correction ($P < 0.05$, $n = 20$ individuals). Locus Ep03 showed significant linkage disequilibrium with locus Ep05 ($P < 0.05$). The nucleotide sequence data will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers provided. 'tail' indicates a tail of the M13f primer (5'-TGTAACGACGGCCAGT'), which is added to the 5'-end of the primer T_a annealing temperature of the primer pair, H_O observed heterozygosity, H_E expected heterozygosity