Development and Characterization of Nine Microsatellites for an Endangered Tree, Pinus wangii (Pinaceae)

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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF NINE MICROSATELLITES FOR AN ENDANGERED TREE, *PINUS WANGII* (PINACEAE)[1]

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- *Premise of the study: Pinus wangii* is an endemic and endangered species in southwestern China, and microsatellite primers were developed to characterize its genetic diversity and population structure.
- *Methods and Results:* Using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol, nine sets of microsatellite primers were developed in *P. wangii*. One population with 26 individuals of *P. wangii*, as well as 11 individuals each for two congeneric species, *P. taiwanensis* and *P. squamata*, were used to test their polymorphism and transferability. The number of alleles per locus ranged from one to seven with an average of 3.7, and the observed heterozygosity and expected heterozygosity ranged from 0 to 0.91 and 0 to 0.75, respectively.
- *Conclusions:* We developed nine sets of polymorphic microsatellite loci that are suitable for investigating genetic diversity and population structure of *P. wangii*, and these markers may be useful for other *Pinus* species.

Key words: microsatellite; Pinaceae; *Pinus wangii*; population genetics.

Pinus wangii Hu & W. C. Cheng is an endemic subtropical tree species in southeastern Yunnan, China. It occurs sparsely in limestone habitats and is narrowly restricted to Xichou and Malipo counties in Yunnan Province (Fu et al.,1999). Due to drastic disturbance of its natural habitat as well as overexploitation for timber, there are only two remaining remnant populations (Fu et al.,1999). Despite the establishment of in situ conservation practices, no studies have measured the genetic diversity and population structure of this critically endangered species, factors which will be useful for assessing its evolutionary potential and establishing concrete conservation strategies. In this study, we developed nine highly variable microsatellite markers for *P. wangii* and tested the transferability of these loci in two congeneric species—*P. taiwanensis* Hayata and *P. squamata* X. W. Li.

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METHODS AND RESULTS

One population of *P. wangii* with 26 individuals was sampled from Xichou County, Yunnan Province (23°10′N, 104°49′E, alt. 1623 m; voucher specimens: DJJ01, deposited at the Herbarium of South China Botanical Garden [IBSC]). In addition, 11 individuals each for two other congeneric species, *P. taiwanensis* (Songyang County, Zhejiang Province; 28°18′N, 119°16′E, alt. 821 m; voucher specimens: DJJ02, IBSC) and *P. squamata* (Qiaojia County, Yunnan Province; 27°08′N, 103°2′E, alt. 2014 m; voucher specimens: DJJ03, IBSC), were used to test the transferability of these primers. Genomic DNA was extracted from silica gel–dried needle samples following the cetyltrimethylammonium bromide (CTAB) method (Doyle, 1991). Using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol developed by Zane et al. (2002), we isolated a set of microsatellite loci from an enriched $(AC)_n$ library. First, ~300 ng of the pooled genomic DNA from three individuals of *P. wangii* was digested with the *Mse*I restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA) overnight at 16°C, then the digested products were ligated to an *Mse*I adapter pair (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) using T4 DNA ligase (Fermentas, Burlington, Ontario, Canada) in a 30 µL reaction mixture for 2 h at 37°C. The 10-fold diluted digestion-ligation mixture was subsequently amplified with the adapter-specific primer *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′) (25 µM). After being denatured at 95°C for 5 min, the former PCR products were hybridized with a 5′-biotinylated probe $([AC]_{15})$ in 250 µL hybridization solution (4× saline sodium citrate [SSC], 0.1% sodium dodecyl sulfate [SDS], 0.5 µmol/L probe) at 48°C for 2 h. Using streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA), the enriched simple sequence repeat (SSR) fragments were separated and captured at room temperature for 30 min, then were subject to an amplification with *Mse*I-N primer, and the amplified PCR products ranging between 300 and 800 bp were gel-sliced by electrophoresis on 1.5% agarose and then purified with the E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Winooski, Vermont, USA). Finally, these recovered DNA

TABLE 1. Characteristics of nine microsatellite loci for *Pinus wangii*.

Locus	Primer sequences (5′–3′)	Repeat motif	Size (bp)	T_a (°C)	GenBank accession no.
PW01	F: CTAATACAACAGCCAATA R: GACATAATGTCTCAGGAT	$(TA)_6(AG)_5$	186	46.0	JQ692298
PW02	F: GGAGCAAATGTGAAGGAC R: AAGGGATTATCAGTCTAAAGAA	$(TG)_{17}(TG)_{16}$	241	47.0	JQ692299
PW03	F: GATGTTGGAACACCTAAT R: GTCAATCACTATCCTTCG	$(TG)_{15}$	284	47.0	JQ692300
PW04	F: GGTACAGTGGCTAACATT R: CCAGAACGACCTACAATC	$(TG)_{17}$	494	50.0	JQ692301
PW05	F: TCCACTAATAGGCTATCG R: ATGCGTATGGAAATTATG	$(TG)_7$	341	53.0	JQ692302
PW06	F: GCGACCTACAGGAACAAC R: TGGAATCCCAGATTACAT	$(TG)_9$	187	48.0	JQ692303
PW07	F: TAGTAGGCTATCGGGTTG R: ATGCATGTGAATGGAAGT	$(TG)_8$	345	53.0	JQ692304
PW08	F: ATTGGAGACATCGGTCAA R: TCAAGTTGCGAGGAGTTT	$(AC)_8$	236	48.0	JQ692305
PW09	F: GCCATTAGGAGGAAGAGG R: CATCCATGCAAATGAAAAT	$(TG)_7$	320	49.0	JQ692306

Note: T_a = optimal annealing temperature.

fragments were transformed and cloned according to a standard clone protocol. A total of 168 positive clones were chosen and sequenced using an ABI 3730 automated DNA Sequencer (Applied Biosystems, Foster City, California, USA). One hundred and two sequences were found to contain microsatellite repeats, of which 50 with sufficient flanking regions were selected to design specific primers using the program Primer Premier version 5.0 (Premier Biosoft International, Palo Alto, California, USA), and candidate primers were subject to the following criteria: GC content varying between 40% and 60%, annealing temperature ranging from 48°C to 60°C, and PCR product size varying between 100 and 400 bp. Nine of the 50 primer pairs were amplified with the appropriate size range and were chosen for polymorphism screening. The forward primers of the nine primer pairs were labeled with fluorescent dye (FAM or TAMRA).

After optimizing the annealing temperature for each pair of primers by gradient PCR (Table 1), PCR amplification was performed in a 20 μL reaction mixture consisting of 20 ng of genomic DNA, 0.2 mM of dNTPs, 0.2 μM of each primer, 1× PCR buffer, and 1 U of *Taq* DNA polymerase (TaKaRa Biotechnology Co., Dalian, China), with the following thermocycling conditions: denaturation for 4 min at 94°C, 35 cycles denaturation at 94°C for 40 s, annealing temperature between 46°C and 53°C (depending on locus) for 45 s, extension at 72°C for 50 s, and a final extension of 8 min at 72°C. Using Genotyper 4.0 with LIZ 500 (Applied Biosystems) as an internal size standard, the fragment sizes of these PCR products were determined on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Nine primer pairs were successfully amplified with expected size ranges (Table 1), and all of them showed polymorphism within populations. Genetic diversity was estimated with POPGENE version 1.3.1 (Yeh et al., 1999). For each locus, the number of alleles (*A*) ranged from one to seven with an average of 3.7; the observed and expected heterozygosities varied from 0 to 0.91 and 0 to 0.75, respectively (Table 2). Except for one locus, PW04, which failed to amplify in *P. taiwanensis*, all of the loci showed good transferability. For all three species, most of the loci demonstrated significant departure from Hardy–Weinberg equilibrium (Table 2) due to a deficiency of heterozygosity. All of the loci should be considered to be independent loci across the genome, because none of them showed significant linkage disequilibrium in the three populations.

CONCLUSIONS

In this study, we developed nine pairs of polymorphic microsatellite primers that are suitable for investigating the genetic diversity and population structure of *P. wangii*. Most of these microsatellite primers can also be applied to two other congeneric species, *P. taiwanensis* and *P. squamata.* We expect these

TABLE 2. Results of initial primer screening in *Pinus wangii* and two congeneric species, *P. taiwanensis* and *P. squamata.*

	P. wangii (*N* = 26)				*P. taiwanensis* (*N* = 11)				*P. squamata* (*N* = 11)			
Locus	*A*	H_o	H_e	HWE[a]	*A*	H_o	H_e	HWE[a]	*A*	H_o	H_e	HWE[a]
PW01	2	0.12	0.18	0.05*	3	0.09	0.26	0.00***	3	0.82	0.60	0.00***
PW02	7	0.46	0.58	0.06^{ns}	6	0.27	0.72	0.00***	3	0.09	0.26	0.00***
PW03	5	0.23	0.40	0.00***	2	0	0.17	0.00***	5	0.36	0.70	0.00***
PW04	5	0.08	0.55	0.00***	—	—	—	—	2	0.18	0.31	0.12^{ns}
PW05	6	0.65	0.75	0.00***	5	0.55	0.58	0.01*	3	0.82	0.65	0.10^{ns}
PW06	4	0.35	0.61	0.06^{ns}	3	0.36	0.39	0.20^{ns}	1	0	0	—
PW07	5	0.69	0.61	0.02*	5	0.36	0.66	0.04*	4	0.82	0.64	0.00***
PW08	4	0.58	0.47	0.69^{ns}	4	0.64	0.50	0.92^{ns}	3	0.91	0.56	0.08^{ns}
PW09	4	0.04	0.47	0.00***	3	0.18	0.39	0.02*	3	0.09	0.26	0.00***

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; *N* = sample size for each population.

[a] Significant departures from HWE: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ns = not significant.

markers will be useful in the conservation genetic study of *P. wangii* as well as other congeneric species.

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