DEVELOPMENT AND CHARACTERIZATION OF NINE MICROSATELLITES FOR AN ENDANGERED TREE, Pinus wangi (Pinaceae)\(^1\)

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- Premise of the study: Pinus wangi 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. wangi. One population with 26 individuals of P. wangi, 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. wangi, and these markers may be useful for other Pinus species.

Key words: microsatellite; Pinaceae; Pinus wangi; population genetics.

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METHODS AND RESULTS

One population of P. wangi 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)\(_2\) library. First, ~300 ng of the pooled genomic DNA from three individuals of P. wangi was digested with the MseI restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA) overnight at 16°C, then the digested products were ligated to an Msel adapter pair (5'-ACTCAGGGACTCAT-3'/5'-GAGGATGAGTCTCCTGAG-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 Msel-N (5'-GATGATGCTCTGTAAN-3') (25 μM). After being denatured at 95°C for 5 min, the former PCR products were hybridized with a 5'-biotinylated probe ([\(\text{AC}\)]\(_2\)) in 250 μL hybridization solution (4x saline sodium citrate [SSC], 0.1% sodium dodecyl sulfate [SDS], 0.5 mM/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 Msel-N primer, and the amplified PCR products ranging between 1000 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
Locus | Primer sequences (5′-3′) | Repeat motif | Size (bp) | T_a (°C) | GenBank accession no.
--- | --- | --- | --- | --- | ---
PW01 | F: CTAATACACAGCCAATA | (TA)_8(AG)_5 | 186 | 46.0 | JQ692298
| R: GACATTAATTCACGAT |  |
PW02 | F: GGCGAAATAGGTGAAGGAC | (TG)_15(TG)_16 | 241 | 47.0 | JQ692299
| R: AAGGGTATTAGTGGGTAAGGA |  |
PW03 | F: GATGTTGAACACCTAAT | (TG)_15 | 284 | 47.0 | JQ692300
| R: GCTAATCATATTCCTGC |  |
PW04 | F: GATCACTGCTCAACATT | (TG)_17 | 494 | 50.0 | JQ692301
| R: CCAGAACGACCTACAATC |  |
PW05 | F: TCCACTAATAGCTATCG | (TG)_17 | 341 | 53.0 | JQ692302
| R: ATGCGTATGGAAATTATG |  |
PW06 | F: GCGACCTACAGAGAACAC | (TG)_9 | 187 | 48.0 | JQ692303
| R: TGGACATCCCTACATTACAT |  |
PW07 | F: TAGTACGGTCATCAGTTTG | (TG)_8 | 345 | 53.0 | JQ692304
| R: ATGCATGTAATGGAAGTT |  |
PW08 | F: ATTCAGGACACCTGCACAA | (AC)_8 | 236 | 48.0 | JQ692305
| R: TCAGTTGGCAGGAGTGTT |  |
PW09 | F: GCCATTCAGGGAAGGAG | (TG)_7 | 320 | 49.0 | JQ692306
| R: CACCTAGCAGGAAATGAAAT |  |

**Note:** T_a = optimal annealing temperature.

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 1.31 (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 0.0 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

| Locus | H_w (N = 26) | H_e (N = 11) | HWE | H_w (N = 11) | H_e (N = 11) | HWE | H_w (N = 11) | H_e (N = 11) | HWE |
|--- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| PW01 | 2 | 0.12 | 0.18 | 0.06** | 3 | 0.09 | 0.26 | 0.00*** | 3 | 0.09 | 0.26 | 0.00*** |
| PW02 | 7 | 0.46 | 0.58 | 0.06** | 6 | 0.27 | 0.72 | 0.00*** | 3 | 0.09 | 0.26 | 0.00*** |
| PW03 | 5 | 0.23 | 0.40 | 0.00*** | 2 | 0.00 | 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*** |
| PW05 | 6 | 0.65 | 0.75 | 0.00*** | 5 | 0.55 | 0.58 | 0.01* | 3 | 0.82 | 0.65 | 0.10*** |
| PW06 | 4 | 0.35 | 0.61 | 0.06** | 3 | 0.36 | 0.39 | 0.20* | 1 | 0.00 | 0.00 | 0.00*** |
| PW07 | 5 | 0.69 | 0.61 | 0.06** | 5 | 0.36 | 0.66 | 0.04* | 4 | 0.82 | 0.64 | 0.00*** |
| PW08 | 4 | 0.58 | 0.47 | 0.69** | 4 | 0.64 | 0.50 | 0.02* | 3 | 0.91 | 0.56 | 0.08** |
| 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_w = expected heterozygosity; H_e = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; N = sample size for each population.

*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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