Development of 11 Microsatellite Loci for an Endangered Herb, Paraisometrum mileense (Gesneriaceae), in Southwest China

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PRIMER NOTE

DEVELOPMENT OF 11 MICROSATELLITE LOCI FOR AN ENDANGERED HERB, *PARAISOMETRUM MILEENSE* (GESNERIACEAE), IN SOUTHWEST CHINA[1]

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- *Premise of the study:* Microsatellite markers were developed to investigate the genetic diversity and population structure of the endangered species *Paraisometrum mileense*, as well as its evolutionary history.
- *Methods and Results:* Using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol, 11 polymorphic primer sets were obtained in *P. mileense*. The number of alleles per locus ranged from one to nine with an average 3.8, and the observed and expected heterozygosities ranged from 0 to 1 and 0 to 0.89, respectively, for the available three populations.
- *Conclusions:* These markers may be useful for further investigation of the conservation genetics of *P. mileense*.

Key words: endangered herb; Gesneriaceae; microsatellite; *Paraisometrum mileense*; population genetics.

Paraisometrum mileense W. T. Wang (Gesneriaceae), a perennial herb from the monotypic genus *Paraisometrum* that was once thought to be extinct, was rediscovered from southeastern Yunnan, China, in 2006, 100 years after the only available specimen was collected (Cai, 2006). *Paraisometrum mileense* inhabits barren and vulnerable limestone habitats, and only three populations have been recorded from Yunnan, Guangxi, and Guizhou provinces (Xu et al., 2009; Gao and Xu, 2011). Due to its close proximity to local settlements, the habitats of *P. mileense* have been disturbed by human activities, and thus this endangered species is at a high risk of extinction. Considering its evolutionary significance in the Gesneriaceae, it is urgent for us to investigate the genetic diversity and demographic history of *P. mileense* populations prior to developing concrete conservation strategies. We developed and characterized 11 novel microsatellite loci for the population genetic study of *P. mileense*.

METHODS AND RESULTS

Twelve individuals of *P. mileense* each from the three populations were sampled in southeastern China (population SL: Shilin County, Yunnan Province, 24°35.175′N, 103°31.947′E, alt. 2012 m; population LL: Longlin County, Guangxi Province, 24°58.6′N, 105°11.2′E, alt. 1245 m; population XY: Xinyi County, Guizhou Province, 24°49.6′N,104°48.5′E, alt. 1452 m). Voucher specimens (TAJ 001 for SL, TAJ 002 for LL, and TAJ 003 for XY) were deposited at the Herbarium of the South China Botanical Garden (IBSC). Three individuals, one from each of the three sampled populations, were pooled for genomic DNA extraction with the cetyltrimethylammonium bromide (CTAB) method (Doyle, 1991). An enriched $(AC)_n$ library of *P. mileense* was constructed following Zane et al. (2002) with minor modifications. First, ~250 ng purified genomic DNA was digested with the *Mse*I restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA) overnight at 16°C, and then ligated to *Mse*I adapters (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) with T4 DNA ligase (Fermentas, Burlington, Ontario, Canada) in a 30 µL reaction mixture. Ten-fold diluted ligation mixture was amplified with adapter-specific primer *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′) (25 µM). After denaturization at 95°C for 5 min, the PCR products were then hybridized with 5′ biotin-labeled oligonucleotide probe $(AC)_{15}$ in a 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. These probe-bound DNA fragments were captured using Streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA) by one incubation at room temperature for 30 min in TEN100, and two rounds of washing steps: nonstringent washing in TEN1000 and subsequent stringent washing (0.2× SSC, 0.1% SDS) for three times. Recovered DNA fragments were amplified with the *Mse*I-N primer, and then purified with the E.Z.N.A Gel Extraction Kit (Omega Bio-Tek, Winooski, Vermont, USA). The purified fragments were ligated into the pMD18-T vector (TaKaRa Biotechnology Co., Dalian, Liaoning, China) and transformed into *E. coli* strain JM109 (TaKaRa Biotechnology Co.). Positive clones were screened out by PCR with primers $(AC)_{15}$ and M13+/ M13−, respectively. A total of 83 positive clones were identified and sequenced on an ABI 3730 automated DNA Sequencer (Applied Biosystems, Foster City, California, USA). Twenty-eight out of 53 clones with microsatellite motifs were used to design specific primers with Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA), and candidate primers with GC contents ranging from 40% to 60%, annealing temperatures varying between 48°C and 60°C, and PCR product sizes ranging from 100 to 400 bp were chosen for subsequent experiments. For those primer pairs with successful amplification, the forward primers were labeled with one of two fluorescent dyes (FAM, TAMRA).

PCR amplifications were performed in 20 µL reaction mixtures containing 20 ng of genomic DNA, 1× PCR buffer, 1 U of *Taq* DNA polymerase (TaKaRa Biotechnology Co.), 0.25 µM of each primer, and 0.25 mM of dNTPs. The thermocycling conditions were: predenaturation for 3 min at 94°C, followed by 35 cycles of 94°C for 45 s, annealing temperature for 45 s, and 72°C for 1 min, and finally a final extension of 7 min at 72°C. Polymorphism detection of these PCR products was implemented on an ABI 3730xl DNA analyzer (Applied Biosystems) using Genotyper 4.0 and LIZ 500 (Applied Biosystems) as an internal size standard.

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TABLE 1. Characteristics of 11 microsatellite loci of *Paraisometrum mileense*.

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	GenBank accession no.
PM01	F: GAAAACGTCCTTCACTTG	$(AC)_{10}$	320–334	48	JQ713323
	R: TGGACACTGGAGAAACAC				
PM02	F: ACAGACGGCGACGGAAAT	$(AG)_6(AC)_5$	136–142	50	JQ713324
	R: ACAGACCGGAGTGTAGGG				
PM03	F: CTCCATCCTCACGCCACCT	$(AC)_7$	124–138	52	JQ713325
	R: GGCAAGCAAACGAGCACC				
PM04	F: TGGCTTCATCTTATCCTT	$(CT)_6(CA)_6$	227–249	48	JQ713326
	R: CTTATTTGGGCTTATAGTTC				
PM05	F: CCTAACCTAATCCCACCA	$(AC)_8$	123–141	49	JQ713327
	R: ACGAACCGATCAACAACT				
PM06	F: GTAAAGCCATGAAACTAACG	$(AC)_7$	265–271	49	JQ713328
	R: TAAACCTACCACCAACCC				
PM07	F: CTATTGATGCTCCGTTGT	$(CA)_7(AT)_6$	242–266	48	JQ713329
	R: GGGGAAAGATATGAAAGT				
PM08	F: CCACCATCACCCTTCCAC	$(AC)_7$	138–158	52	JQ713330
	R: ACAGCAAGCAAACGAGCA				
PM09	F: GCTTGATGGAATGAGGTT	$(AC)_6$	160–162	46	JQ713331
	R: ATTGACTTCACTTGTGCG				
PM10	F: AGACGGCGACAGAAATAG	$(TC)_6$	131–145	49	JQ713332
	R: ACAGACCGAAATGTAGGG				
PM11	F: TCTCCTACCACTCCCACG	$(AC)_8$	140–144	51	JQ713333
	R: GACAAGCAAACGAGCACC				

Note: T_a = optimal annealing temperature.

TABLE 2. Results of initial primer screening in three populations of *Paraisometrum mileense*.

	Xinyi (N = 12)				Shilin (N = 12)				Longlin (N = 12)			
Locus	A	H_o	H_e	HWE[a]	A	H_o	H_e	HWE[a]	A	H_o	H_e	HWE[a]
PM1	4	0.17	0.37	0.02*	4	0.42	0.37	0.99 n.s.	3	0.17	0.16	0.99 n.s.
PM2	3	0	0.30	0.00***	3	0	0.42	0.00***	2	0	0.16	0.00***
PM3	7	0.92	0.83	0.00***	2	1	0.52	0.00***	3	1	0.56	0.01*
PM4	5	0.42	0.69	0.04*	2	0	0.16	0.00***	4	0.50	0.69	0.37 n.s.
PM5	5	0.08	0.50	0.00***	3	0.08	0.24	0.00***	4	0.17	0.71	0.00***
PM6	4	0.17	0.47	0.03*	1	0	0	—	1	0	0	—
PM7	7	0.92	0.80	0.00***	5	0.58	0.70	0.09 n.s.	4	0.58	0.65	0.00***
PM8	6	0.33	0.80	0.00***	9	1	0.89	0.09 n.s.	5	0.50	0.71	0.01*
PM9	1	0	0	—	2	0.75	0.49	0.05 n.s.	1	0	0	—
PM10	6	0.75	0.78	0.73 n.s.	7	0.75	0.84	0.10 n.s.	4	1	0.66	0.02*
PM11	3	0.67	0.68	0.00***	3	0.08	0.16	0.00***	2	0.83	0.52	0.03*

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; n.s. = not significant.

Eleven primer pairs were successfully amplified with the expected sizes based on the corresponding clones (Table 1). Genetic diversity parameters, departure from Hardy–Weinberg equilibrium (HWE), and linkage disequilibrium (LD) between pairs of loci were estimated with POPGENE version 1.3.1 (Yeh et al., 1999). The number of alleles per loci (A) ranged from one to seven with an average of 3.8 for each population, and the observed and expected heterozygosities ranged from 0 to 1 and from 0 to 0.89, respectively. All of the 11 microsatellite loci showed clear polymorphic patterns. Most of the loci exhibited significant departure from HWE in all three populations (Table 2), due to a deficiency of heterozygosity. None of the loci showed significant LD among the three populations, and they should be considered to be independent loci across the genome.

CONCLUSIONS

The 11 polymorphic microsatellite markers are valuable tools for the conservation genetics of this highly endangered herb. Population genetic studies based on these markers are expected to reveal the population structure, effective population size, mating system, gene flow between the remnant populations, and provide fundamental genetic information for making concrete conservation strategies.

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