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Primer Note

Development of microsatellite markers for *Fargesia denudata* (Poaceae), the staple-food bamboo of the giant panda[1]

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- *Premise of the study:* There is a need for microsatellite primers to analyze genetic parameters of *Fargesia denudata* (Poaceae), the staple-food bamboo of the giant panda (*Ailuropoda melanoleuca*).
- *Methods and Results:* Using next-generation sequencing technology, we obtained a 75-Mb assembled sequence of *F. denudata* and identified 182 microsatellites. Primer pairs for 70 candidate microsatellite markers were selected and validated in four individuals, and 42 primer pairs generated reliable amplicons. Fourteen of 16 tested markers were found to be polymorphic in 72 individuals from four *F. denudata* populations. The number of alleles ranged from two to 19 per locus; the observed and expected heterozygosities ranged from 0 to 1 and from 0 to 0.87, respectively. The transferability of these 16 novel microsatellite markers was validated in five related species.
- *Conclusions:* These markers will be useful for examining the genetic diversity, genetic structure, and cloning of *F. denudata*, the staple-food bamboo of the giant panda, and related bamboo species.

Key words: *Fargesia denudata*; giant panda; microsatellites; next-generation sequencing; Poaceae.

The giant panda (*Ailuropoda melanoleuca*) is a flagship species in nature conservation (Swaisgood et al., 2010). One of its biological and behavioral characteristics is a highly restricted diet, consisting mainly of bamboo (Christian et al., 2015). The most important bamboo species for the giant panda's diet is *Fargesia denudata* T. P. Yi (Poaceae), which is endemic to China (Zeng et al., 2002). *Fargesia denudata* has four growth stages for aboveground parts: the slow growth stage, the exponential stage, the relative growth decreasing stage, and the dormant stage. The fastest growth stage is the exponential stage, which contributes 92% of the annual biomass production and has a maximum daily height growth increment of up to 6.1 cm (Wang et al., 1991). In spring, the most active part of *F. denudata* grows underground, where basal buds germinate. *Fargesia denudata* mainly occurs in Wanglang National Nature Reserve (WNNR), one of four main reserves established to protect the giant panda, other rare wild animals, and their habitats (http://www.wanglang.com/), located in the northern part of Pingwu County, Sichuan Province, China (32°49′–33°02′N, 103°50′–104°58′E) (Li and Shen, 2012). However, *F. denudata* died across a large area after flowering in the 1970s, seriously threatening the giant panda's survival (Qin, 1985). Furthermore, despite its ecological significance there have been no genetic studies of *F. denudata*, mainly due to a lack of appropriate species-specific molecular markers. Thus, we have developed 16 microsatellite markers for *F. denudata* using next-generation sequencing technology and evaluated their transferability to five other related species.

METHODS AND RESULTS

Genomic DNA was extracted from silica gel–dried leaves of *F. denudata*, sampled in WNNR, using a DNAquick Plant System following the manufacturer's protocol (TIANGEN, Beijing, China). Vouchers were deposited at Beijing Forestry University (Appendix 1). A 500-ng portion of purified genomic DNA product was used for library construction, via a 126-bp paired-end run on a HiSeq 1500 system (Illumina, San Diego, California, USA), part of the sequencing platform of the School of Life Sciences, Tsinghua University, China. Briefly, the manufacturer's instructions to obtain 400-bp inserts were followed, using RTA version 1.6 (Illumina) for base-calling and in-house Perl scripts to remove weak signals and low-quality sequences (Du et al., 2015). Read ends were further screened and trimmed used DynamicTrim.pl and LengthSort.pl in SolexaQA (Cox et al., 2010). In total, 1.6 GB of raw data were obtained and 2,992,449 reads were accepted after removing reads with a base error rate >0.1% and/or <50 bp long. De novo sequence assembly by SOAPdenovo2 (Luo et al., 2012) cannot provide enough contiguity for further analysis, thus we switched to reference-guided assembly (RGA) using the draft genome of *Phyllostachys heterocycla* (Carrière) S. Matsum. cv. Pubescens as the reference genome. We obtained 424,321 contigs, of which the longest contig is 2454 bp and 125 contigs had a length >1 Kb. Simple sequence repeats (SSRs) were detected using the MIcroSAtellite identification tool (MISA; Thiel et al., 2003). We searched for mono-, di-, tri-, tetra-, penta-, and hexanucleotide repeats, with repeat counts ≥10 for mononucleotides and ≥6 for the other repeats, and interruption (max_difference_for_2_SSRs) of 100 bp. We found 182 microsatellites meeting these criteria (Appendix S1) and developed primer pairs in flanking

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regions using Primer3 (Rozen and Skaletsky, 1999) for all of the identified SSRs with annealing temperatures ranging from 50°C to 58°C and fragment sizes from 100 to 300 bp.

We randomly selected 70 di- and trinucleotide microsatellites from these pairs for further validation in four individuals randomly selected from four populations (one individual from each population). For primary microsatellite screening, PCR was performed in a 25-μL reaction volume containing 1× *Taq* buffer, 0.2 mM dNTPs, 10–20 ng template DNA, 1.6 pM of forward and reverse primer each, and 1 unit *Taq* polymerase (Aidlab Biotechnologies Co., Ltd., Beijing, China). Conditions of the PCR were: 94°C for 5 min; 30 cycles of 94°C for 30 s, 56°C for 45 s, 72°C for 45 s; and a final extension at 72°C for 10 min. Five microliters of the PCR products were separated on 2% agarose gels to check for successful amplification. Forty-two of the 70 microsatellites were successfully amplified, and the presence of repeated motifs in 16 of them was confirmed by Sanger sequencing (Table 1). All of the confirmed microsatellite sequences were deposited at the National Center for Biotechnology Information (NCBI; accession no. KU043295–KU043310) (Table 1).

DNA samples from 72 individuals of four populations were used to test the polymorphism of the 16 markers using a rapid and inexpensive method based on the M13-tail technique (Schuelke, 2000; Du et al., 2013). Briefly, a 5′ M13-tailed forward primer, a reverse primer, and a fluorescent-labeled M13 primer were synthesized for each genotyping run and the M13 primers were labeled with FAM, HEX, ROX, and TAMRA (Sangon Biotech, Shanghai, China), respectively. Each 15-μL PCR contained 5–20 ng template DNA, 1× *Taq* mix (Aidlab Biotechnologies Co., Ltd.), 2.4 pM of the reverse and fluorescent-labeled M13 primers, and 0.6 pM of the forward primer. The PCR conditions were: 94°C for 5 min; then 30 cycles of 94°C for 30 s, 56°C for 45 s, 72°C for 45 s; followed by eight cycles of 94°C for 30 s, 53°C for 45 s, 72°C for 45 s; and a final extension at 72°C for 10 min. For each PCR product with the four different fluorescent dyes, 0.5 μL were mixed and added to 10 μL of formamide and 0.5 μL of GeneScan 500 LIZ Size Standard (Applied Biosystems, Waltham, Massachusetts, USA) and run on an ABI PRISM 3730 Genetic Analyzer. Raw data were analyzed with GeneMarker version 1.75 (SoftGenetics, State College, Pennsylvania, USA).

The total number of alleles, observed heterozygosity (H_o), expected heterozygosity (H_e), and χ^2 probabilities of Hardy–Weinberg equilibrium (HWE) for 14 of the loci found to be polymorphic were determined using GenAlEx 6.41 (Peakall and Smouse, 2006). The number of alleles ranged from two to 19 per locus, H_o from 0 to 1, H_e from 0 to 0.87, and the χ^2 tests revealed that three loci (FAD012, FAD018, and FAD049) were not in HWE (Table 2), mainly because of clonal propagation. No linkage disequilibrium was detected in these loci by GENEPOP 4.2 (Rousset, 2008).

We chose five bamboo species (*Arundinaria fargesii* E. G. Camus, *Yushania lineolata* T. P. Yi, *Fargesia scabrida* T. P. Yi, *F. rufa* T. P. Yi, and *F. ferax* (Keng) T. P. Yi) to test the transferability of the 16 markers. Vouchers were deposited at Beijing Forestry University (Appendix 1). DNA of five individuals from each of these species was subjected to PCR amplification using the 16 novel microsatellite primers identified in *F. denudata*, and PCR products were separated by 2% agarose gel electrophoresis followed by GoldView staining. The overall transferability of the 16 microsatellite markers proved to be 95.50%, ranging from 90.00% for *Y. lineolata* to 98.75% for *F. rufa* and *F. ferax* (Table 3).

CONCLUSIONS

The novel microsatellite markers developed in this study will be useful for exploring the genetic status of *F. denudata*, the giant panda's staple-food bamboo, as well as other related bamboo species. Moreover, analysis of the genetic dynamics in key areas using these polymorphic markers should assist efforts to conserve important bamboo populations in the giant panda's habitat.

Table 1. Characteristics of 16 microsatellite loci developed for *Fargesia denudata.*

Locus	Primer sequences (5′–3′)	Repeat motif	Fluorescent dye[a]	Allele size (bp)	T_a (°C)	*A*	GenBank accession no.
Fad002	F: TACCAGGGAACAAGGCGA	$(GT)_6$	HEX	150	57	10	KU043310
	R: CTGCGCCTGTCTCCGTAT						
Fad006	F: CACAAGCGTAGTTTTGCCAG	$(TG)_6$	ROX	170	55	4	KU043309
	R: TGCTCAAGAGCCACCAAA						
Fad008	F: TGTGCAATTTTGCTTCATACA	$(CA)_4$	HEX	170	50	9	KU043308
	R: TTCCTTTGCCTTATGCCG						
Fad011	F: ATCAAGGCGCAGGATGAG	$(CT)_5$	TAMRA	200	56	4	KU043307
	R: ACGTCTAGCTGGTTGCGG						
Fad012	F: AGCCCAGTCCATCACCAA	$(CT)_5$	HEX	270	58	4	KU043306
	R: GAGGCCGGTCCTTGAAGT						
Fad016	F: GCGACACCGTCCTCAACT	$(AG)_7$	FAM	230	55	7	KU043305
	R: TCGTGACAAAGCCTGGAA						
Fad018	F: ACGAACCAGCTGCCGTAG	$(CAC)_4$	ROX	280	57	2	KU043304
	R: CCATGGACCAGGTCTTCG						
Fad023	F: CATGGTTAATGGCCTCCG	$(GCG)_6$	HEX	220	55	8	KU043303
	R: CTTGAGAGGGAGGAGGGG						
Fad038	F: ACTGAGGGGGAGCAGGAC	$(CT)_5$	FAM	220	57	1	KU043302
	R: GCCATCTTGTGGGGTACG						
Fad042	F: CTGGAGTCGCTCCACGAT	$(AG)_5$	ROX	170	58	1	KU043301
	R: CTGAGGGGGAGCAAGACA						
Fad044	F: CCCCTCTCTCCAAGCTCC	$(AG)_{11}$	HEX	250	56	19	KU043300
	R: TCGCTTTCTCGTCGATCC						
Fad046	F: TTCGCCCTCTTCAAGGTG	$(AG)_3$	TAMRA	150	56	4	KU043299
	R: CTTTCTTGCCGACTCCGA						
Fad049	F: TGCTCCGCAAGAGCCTAC	$(GTC)_4$	FAM	210	57	5	KU043298
	R: GCTCGAATGCCATCAGGT						
Fad050	F: TGGAGTAGGCTCTTGCGG	$(TC)_6$	HEX	240	55	5	KU043297
	R: CGTCGCGACATGCTTATG						
Fad053	F: ACCCTTGCGTTGGCTACA	$(AAG)_5$	ROX	150	56	2	KU043296
	R: AAGGCGAGGGCAAAAGAT						
Fad068	F: CCTGTCCCTTGTCGCGTA	$(CG)_6$	TAMRA	70	58	3	KU043295
	R: AAAAGTCCACGGGGTGGT						

Note: *A* = total number of alleles based on the initial screen of 72 individuals; T_a = annealing temperature.
[a]Fluorescent dye on forward primers.

TABLE 2. Fourteen polymorphic microsatellite locus–specific measures of genetic diversity across four populations of *Fargesia denudata*.[a]

	Pop 1 (n = 24)				Pop 2 (n = 24)				Pop 3 (n = 12)				Pop 4 (n = 12)				Total (n = 72)		
Locus	A	H_o	H_e	P^b	A	H_o	H_e	P^b	A	H_o	H_e	P^b	A	H_o	H_e	P^b	A	H_o	H_e
Fad002	2	0.04	0.04	0.917	3	0.08	0.22	<0.01	5	0.33	0.36	0.308	5	0.42	0.42	0.290	10	0.17	0.55
Fad006	3	0.92	0.58	<0.001	4	0.96	0.61	<0.001	2	1.00	0.50	<0.001	4	1.00	0.57	0.062	4	0.96	0.58
Fad008	5	0.54	0.47	0.978	7	0.79	0.68	0.945	4	0.50	0.41	0.970	4	0.50	0.42	0.970	9	0.61	0.74
Fad011	1	0.00	0.00	M	1	0.00	0.00	M	2	0.33	0.28	0.488	3	0.33	0.29	0.923	3	0.11	0.47
Fad012	4	0.13	0.16	<0.001	1	0.00	0.00	M	1	0.00	0.00	M	1	0.00	0.00	M	4	0.04	0.05
Fad016	5	0.50	0.74	0.106	6	0.50	0.67	0.146	4	0.92	0.71	0.202	5	1.00	0.69	0.165	7	0.65	0.75
Fad018	2	1.00	0.50	<0.001	2	0.96	0.50	<0.001	2	1.00	0.50	<0.001	2	1.00	0.50	<0.001	2	0.99	0.50
Fad023	7	0.75	0.80	0.723	8	0.92	0.80	0.764	5	0.67	0.66	0.842	6	0.58	0.78	0.107	8	0.76	0.81
Fad044	11	0.88	0.87	0.586	14	0.96	0.85	0.997	8	0.58	0.84	<0.05	12	0.75	0.85	0.228	19	0.83	0.90
Fad046	2	0.08	0.08	0.831	3	0.25	0.23	0.921	2	0.08	0.08	0.880	1	0.00	0.00	M	4	0.13	0.52
Fad049	3	0.92	0.52	<0.001	4	0.83	0.53	<0.05	4	0.92	0.60	0.198	3	0.92	0.53	<0.05	5	0.89	0.54
Fad050	4	0.13	0.12	1.000	2	0.04	0.04	0.917	3	0.17	0.16	0.992	1	0.00	0.00	M	5	0.08	0.08
Fad053	1	0.00	0.00	M	2	0.04	0.04	0.917	2	0.42	0.33	0.362	2	0.25	0.22	0.621	2	0.13	0.41
Fad068	1	0.00	0.00	M	2	0.04	0.04	0.917	2	0.08	0.08	0.880	1	0.00	0.00	M	3	0.03	0.46

Note: A = number of alleles sampled; H_e = expected heterozygosity; H_o = observed heterozygosity; M = monomorphic; n = number of individuals sampled.
[a] Geographic coordinates of the populations are provided in Appendix 1.
[b] P values: χ^2 probabilities of Hardy–Weinberg equilibrium.

TABLE 3. Transferability of the 16 novel microsatellite markers across five related Arundinarieae species (Poaceae).

Locus	*Fargesia scabrida*					*Fargesia rufa*					*Fargesia ferax*					*Arundinaria fargesii*					*Yushania lineolata*					Successful amplifications (%)
Fad002	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	+	+	+	+	+	96
Fad006	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Fad008	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Fad011	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Fad012	+	+	+	+	+	+	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	88
Fad016	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	60
Fad018	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Fad023	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Fad038	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Fad042	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	96
Fad044	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Fad046	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Fad049	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	+	+	+	+	+	+	+	+	+	96
Fad050	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	+	+	+	+	+	+	92
Fad053	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Fad068	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100

Note: + successful amplification; — = unsuccessful amplification.

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Appendix 1. Geographic locations and voucher information for specimens used in this study.

Species	Voucher specimen accession no.[a]	Collection locality	Geographic coordinates
Fargesia denudata T. P. Yi	Fd-Pop1-YL	WNNR, Sichuan, China	32°0′0.144″N, 104°2′51.324″E
	Fd-Pop2-YL	WNNR, Sichuan, China	33°0′39.528″N, 104°1′32.844″E
	Fd-Pop3-YL	WNNR, Sichuan, China	32°55′2.748″N, 104°7′37.092″E
	Fd-Pop4-YL	WNNR, Sichuan, China	32°59′10.572″N, 104°3′45.396″E
Fargesia scabrida T. P. Yi	Fs-YL	TNR, Sichuan, China	32°37′16.064″N, 104°50′39.059″E
Fargesia rufa T. P. Yi	Fr-YL	TNR, Sichuan, China	32°35′49.668″N, 104°49′42.204″E
Fargesia ferax (Keng) T. P. Yi	Ff-YL	TNR, Sichuan, China	29°49′15.139″N, 102°23′19.568″E
Yushania lineolata T. P. Yi	Yl-YL	TNR, Sichuan, China	29°0′21.866″N, 102°23′52.670″E
Arundinaria fargesii E. G. Camus	Af-YL	TNR, Sichuan, China	32°49′49.368″N, 104°49′49.368″E

Note: TNR = Tangjiahe Nature Reserve; WNNR = Wanglang National Nature Reserve; YL = Yan Lv, collector.
[a]Vouchers deposited at the College of Forestry, Beijing Forestry University, Beijing, China.