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PRIMER NOTE

DEVELOPMENT OF POLYMORPHIC MICROSATELLITE MARKERS FOR *PHYLLOSTACHYS EDULIS* (POACEAE), AN IMPORTANT BAMBOO SPECIES IN CHINA[1]

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- *Premise of the study:* Polymorphic microsatellite markers were developed for *Phyllostachys edulis* (Poaceae), an ecologically and economically important bamboo species in China, to evaluate the genetic diversity and population genetic structure of *P. edulis* and other *Phyllostachys* species.
- *Methods and Results:* Twenty microsatellite markers were developed and their polymorphisms were tested on 71 samples from three geographically disparate populations. Each locus exhibited between two and 10 alleles with an average of five alleles. Excluding monomorphic loci, observed and expected heterozygosity ranged from zero to one and from 0.041 to 0.676, respectively.
- *Conclusions:* These 20 polymorphic microsatellite loci will be useful for studies on the molecular ecology, population genetics, and conservation of *P. edulis*.

Key words: bamboo; microsatellite; *Phyllostachys edulis*; Poaceae; population genetics.

Phyllostachys edulis (Carrière) J. Houz. (Poaceae) is the most ecologically and economically important bamboo species in China, and accounts for more than 70% of commercially planted bamboo (Fu, 2001). Because of its wide commercial value, this bamboo was widely cultivated in past decades, which unavoidably decreased the range of natural bamboo stands. Therefore, currently the investigation of gene diversity and preservation of genetic resources are crucial issues. Microsatellite markers are increasingly used for understanding population genetics and evolution (Thomson et al., 2010). A few simple sequence repeat (SSR) markers have been reported for this bamboo in recent years; however, they were only applied for interspecies identification (Tang et al., 2010). To date, studies on the population genetics of *P. edulis* are still rare because of limited codominant markers. The 20 polymorphic SSRs presented in our study will be valuable for determining the molecular ecology and population genetic structure in *P. edulis*.

METHODS AND RESULTS

In total, 10,608 cDNAs of *P. edulis* were downloaded from the National Center for Biotechnology Information (NCBI) database. A total of 425 SSRs ≥20 nucleotides in length (unit/minimum number of repeats: 2/10, 3/7, 4/5, 5/4) were identified from nonredundant *P. edulis* cDNAs using the Simple Sequence Repeat Identification Tool (SSRIT; http://www.gramene.org/db/markers/ssrtool; Temnykh et al., 2001). Primer Premier 5 software (PREMIER Biosoft International, Palo Alto, California, USA) was used to successfully design 191 primer pairs against the sequences flanking each SSR according to these criteria: optimum annealing temperature ranging from 52°C to 62°C; maximum of 3°C difference in annealing temperature between primer pairs; GC content of 40–60%; and PCR product size of 100–380 bp.

To characterize microsatellite loci polymorphisms, we genotyped 71 individuals from three natural populations in China, including Renhua (RH), Guizhou (25°7′–21′N, 113°48′–58′E); Jianou (JO), Fujian (26°58′–27°9′N, 118°13′–18′E); and Xianning (XN), Hubei (29°37′–48′N, 114°10′–18′E). The interval between samples, to avoid being from the same genet, was at least 1 km in every population. Voucher specimens were deposited at the Herbarium of Nanjing Forestry University (Appendix 1). Total genomic DNA was extracted from silica gel–dried young leaves using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990) with minor modifications. PCR was performed in a 20-μL reaction volume containing 50–70 ng of template DNA, 2 μL of 10× PCR buffer, 0.1 mM dNTPs, 0.87 mM $MgCl_2$, 0.48 μM of each primer, and 1 unit of *Taq* DNA polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan). All PCR reactions were performed in an Eppendorf Mastercycler gradient PCR thermal cycler (Eppendorf, Hamburg, Germany) using a modified touchdown protocol (Don et al., 1991): 94°C for 5 min; 12 cycles of 94°C for 30 s, 62°C decreasing to 50°C at 1°C per cycle for 30 s, 72°C for 30 s; 20 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 5 min. After prescreening 12 individuals (randomly sampled from the three populations), 20 of 191 microsatellite loci were identified based on their PCR results and obviously variable bands detected in an 8% denaturing polyacrylamide gel with silver nitrate.

The final 20 loci were 5′ end-labeled using a forward primer with 6-FAM or 6-HEX and genotyped for three populations (Tables 1 and 2). All PCR products were separated with GeneScan 500 ROX Size Standard on an ABI 3730xL DNA analyzer (Applied Biosystems, Carlsbad, California, USA), and fragment sizes were estimated with GeneMapper version 4.0 (Applied Biosystems). Number of observed alleles (*A*), observed and expected heterozygosity (H_o and H_e), Shannon's information index (*I*), deviations from Hardy–Weinberg equilibrium (HWE), and linkage disequilibrium (LD) between loci were estimated using GenAlEx version 6.5 (Peakall and Smouse, 2012).

All loci displayed polymorphisms when compared across populations, with the total *A* ranging from two to 10 alleles per locus and an average of

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TABLE 1. Characteristics of 20 polymorphic microsatellite loci for *Phyllostachys edulis*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Fluorescent label	Allele size (bp)	GenBank accesssion no.
Phe01	F: CACCTCTTTCGTCATCAACC	$(AG)_{29}$	6-FAM	219–255	FP093322
	R: ATCTAACGGCCCAAATGC				
Phe10	F: TAAGGCCCACGTTGCCAG	$(AG)_{19}$	6-FAM	191–227	FP095585
	R: CGCTGAAATCCACCCAGAAG				
Phe13	F: TCGCCATCCCTTATCCAC	$(CT)_{17}$	6-HEX	160–178	FP096712
	R: GCAACGACGCACCTCCTA				
Phe23	F: CCCCATGTTTACCTATCCC	$(TC)_{14}$	6-HEX	365–389	FP091611
	R: GCATCCTCTTGCGCTTAC				
Phe24	F: ACATACCCGCACCACCAA	$(AG)_{14}$	6-HEX	119–125	FP092058
	R: CGACCACCTCGCAAACAA				
Phe28	F: CCTCCGATGAAGCTGAAC	$(TC)_{14}$	6-FAM	243–261	FP096429
	R: CGGGTCCTTGGACAAACT				
Phe32	F: CCTCAAGGCCAGGGTAAG	$(CT)_{13}$	6-FAM	96–112	FP092440
	R: CTCCGTTTCTTTGGTTTGTT				
Phe34	F: GTCGCTCCTCAGTCCTCACA	$(AG)_{13}$	6-HEX	159–171	FP096264
	R: TCCTGCTCCAGGTATTCGTAA				
Phe35	F: AACCACCTCATCACCCACA	$(AG)_{12}$	6-HEX	212–226	FP093046
	R: GCTTTGCACCCTTTATTGCT				
Phe37	F: GCTCTTCGCCAAGTGCTAC	$(CT)_{12}$	6-HEX	196–213	FP094642
	R: GGGACCCATGCCTGTTCTA				
Phe40	F: AGGTTCGTGTTCCGTGGGT	$(GA)_{12}$	6-FAM	107–111	FP097227
	R: TTAGGCGCAGGAAGGTTGG				
Phe44	F: ACTGCGAGGTTCGTGTTC	$(GA)_{12}$	6-FAM	152–166	FP099997
	R: GTAAAGGTTTGACGGGTAGA				
Phe51	F: GTCGCCGTCTCAAGGAGT	$(CT)_{11}$	6-HEX	158–168	FP093298
	R: GTTGCACCATCGGGATTA				
Phe98	F: TCTCCATGCGAATGTGAT	$(CCA)_8$	6-FAM	168–189	FP094032
	R: CGTCTAGTGCTAGGGTTTGT				
Phe100	F: GACATTAGGCGAGGTTCGG	$(CTT)_8$	6-HEX	189–204	FP094809
	R: GGGAGATGGACAGGTTTGCT				
Phe139	F: TTCCTTCTCGCCGCAAAT	$(GCC)_7$	6-FAM	168–195	FP096112
	R: GGCTTGGGATTGAGACTGG				
Phe141	F: AGGCCATAAGGAACTGCT	$(CGT)_7$	6-HEX	321–336	FP096517
	R: GCTTCCAAACCTCCCATC				
Phe163	F: CTAACAAAACAAATCCCCATC	$(CGC)_7$	6-FAM	110–125	FP099798
	R: TCCATCGCGTATTCCACC				
Phe167	F: AACAGCGAAACCACAGACC	$(CCTG)_7$	6-FAM	151–163	FP100624
	R: AGCAGGATGAGACGAGCC				
Phe185	F: TGTAAGTACCCTGCCTCCG	$(CAAT)_5$	6-HEX	145–163	FP097509
	R: GCTGTCTCCCTTCTTCCTG				

[a]All loci were amplified with the same touchdown protocol with initial annealing temperature of 62°C and final annealing temperature of 52°C.

five alleles. Excluding monomorphic loci, H_o and H_e were from 0 to 1 and from 0.041 to 0.676, respectively, while I ranged from 0.101 to 1.443 (Table 2). A total of 14 loci significantly ($P < 0.005$) deviated from HWE in all three populations, and there was no significant LD among all pairs of loci. Similar results were reported in other bamboo species (Kaneko et al., 2008; Miyazaki et al., 2009). The deviation at 14 loci was possibly caused by nonrandom mating within populations due to the unique biological characteristics of bamboo species, such as their highly clonal propagation, monocarpic nature with gregarious flowering, and long flowering intervals (67–120 yr) (Janzen, 1976; Watanabe et al., 1982), as well as the decreasing size of wild populations.

CONCLUSIONS

Twenty novel microsatellite loci showed a useful degree of polymorphism at the population level and will be helpful for molecular ecological studies of *P. edulis*, such as clonal identification, genetic structure, the evolution of gregarious flowering behavior, as well as for elucidating the biogeographic history of this bamboo species.

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Table 2. Polymorphism analyses using 20 markers in three geographically disparate populations of *Phyllostachys edulis*.[a]

	RH (n = 23)				JO (n = 24)				XN (n = 24)			
Locus	A (pA)	H_o	H_e	I	A (pA)	H_o	H_e	I	A (pA)	H_o	H_e	I
Phe01	4	0.652	0.676*	1.234	4	0.417	0.481*	0.888	6 (2)	0.125	0.364*	0.836
Phe10	6 (2)	0.130	0.612*	1.233	4 (1)	0.042	0.261*	0.555	5 (2)	0.042	0.511*	1.004
Phe13	5 (1)	0.000	0.571*	1.119	3	0.042	0.284*	0.513	4	0.042	0.350*	0.679
Phe23	5	0.217	0.538*	1.092	3	0.042	0.081*	0.202	6	0.125	0.332*	0.779
Phe24	4 (1)	0.130	0.339*	0.702	2	0.000	0.080*	0.173	3	0.083	0.226*	0.456
Phe28	4	0.304	0.559*	1.022	4	0.042	0.228*	0.503	4	0.083	0.490*	0.882
Phe32	7 (4)	0.087	0.672*	1.443	2	0.000	0.080*	0.173	3 (1)	0.042	0.155*	0.334
Phe34	3 (1)	0.000	0.510*	0.876	3	0.042	0.081*	0.202	4 (1)	0.083	0.435*	0.752
Phe35	5 (3)	0.130	0.489*	1.011	1	—	—	—	3 (1)	0.042	0.227*	0.463
Phe37	3	0.609	0.631	1.047	3	1.000	0.520*	0.780	3	1.000	0.569*	0.918
Phe40	3	0.087	0.355*	0.632	3	0.042	0.254*	0.475	1	—	—	—
Phe44	2	0.217	0.194	0.344	1	—	—	—	2	0.042	0.041	0.101
Phe51	4 (2)	0.000	0.427*	0.838	2	0.000	0.153*	0.287	3 (1)	0.000	0.226*	0.456
Phe98	4 (1)	0.043	0.542*	0.982	3	0.042	0.155*	0.334	4	0.042	0.157*	0.373
Phe100	5	0.435	0.583*	1.175	4	0.125	0.228*	0.503	5	0.167	0.326*	0.698
Phe139	2	0.696	0.454	0.646	3	1.000	0.520	0.780	5 (2)	0.917	0.554	0.946
Phe141	4 (1)	0.391	0.578*	1.032	4 (2)	1.000	0.540*	0.866	3	0.958	0.539*	0.837
Phe163	2 (1)	0.130	0.122	0.241	2	0.042	0.041	0.101	3 (1)	0.042	0.119*	0.274
Phe167	2	0.696	0.454	0.646	2	0.958	0.499*	0.692	2	0.833	0.486*	0.679
Phe185	3 (1)	0.739	0.509	0.771	3	1.000	0.520*	0.780	3	0.917	0.515	0.774

Note: — = monomorphic loci; A = number of different alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; I = Shannon's information index; pA = number of private alleles per population.

* Significant Hardy–Weinberg disequilibrium ($P < 0.005$).

[a] All populations located in China, see Appendix 1 for locality information.

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Appendix 1. Voucher specimens of *Phyllostachys edulis* used in this study. All vouchers are deposited in the Herbarium of Nanjing Forestry University, Nanjing, Jiangsu, China.

Code	Collection locality	Latitude	Longitude	Voucher no.
RH	Renhua, Guangdong Province	25°7′–21′N	113°48′–58′E	PheRH201106
JO	Jianou, Fujian Province	26°58′–27°9′N	118°13′–18′E	PheJO201108
XN	Qian Mountain, Hubei Province	29°37′–48′N	114°10′–18′E	PheXN201107