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Sixteen novel microsatellite markers developed for *Dendrocalamus sinicus* (Poaceae), the strongest woody bamboo in the world[1]

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- *Premise of the study:* Microsatellite primers were developed in the strongest and most economically important bamboo species, *Dendrocalamus sinicus*, to investigate its genetic variability.
- *Methods and Results:* Using the Fast Isolation by AFLP of Sequences COntaining Repeats (FIASCO) protocol, 16 polymorphic primer sets were identified within 24 individuals from two geographic locations. The number of alleles per locus ranged from two to four, with a mean of 2.6. The observed and expected heterozygosities varied from 0 to 1.000 and from 0.311 to 0.754, respectively. The cross-amplification of the 16 primer pairs was tested in four other *Dendrocalamus* species, and nine to 13 (56.3–81.3%) primer pairs were successfully amplified.
- *Conclusions:* These markers will be useful for studies on the genetic diversity of *D. sinicus*, which may facilitate conservation of this species.

Key words: *Dendrocalamus sinicus*; genetic diversity; genetic variation; microsatellite markers; Poaceae.

As the strongest woody bamboo documented in the world to date, *Dendrocalamus sinicus* L. C. Chia & J. L. Sun can grow to more than 30 m in height and 30 cm in diameter (Li et al., 2004; Hui et al., 2006). *Dendrocalamus sinicus* is endemic to southern and southwestern Yunnan Province, China, where it is economically important as a raw material for furniture, construction, and industrial paper pulp (Yi et al., 2008). Two main types of culm shape (i.e., bending culmed and straight culmed) are found in the southern and northern distribution areas of *D. sinicus*, respectively (Li et al., 2004; Hui et al., 2006). In addition, many *D. sinicus* clumps, which bear both bending and straight culms, occur in the middle of habitats of this species (Gu et al., 2012). Plants with bending culms are difficult to process for producing bamboo timbers; therefore, this type is not suitable to popularize for cultivation (Hui et al., 2006). At present, the development and utilization of *D. sinicus* have been seriously hampered by its small-scale resources and prominent differentiation among provenances (Hui et al., 2006). The aim of this work is to describe the genetic variability and to identify provenances of *D. sinicus*, and therefore to contribute to its conservation and breeding programs. In the current study, we (1) developed microsatellite markers for *D. sinicus*, and (2) performed cross-amplification tests in four other *Dendrocalamus* species, i.e., *D. latiflorus* Munro, *D. hamiltonii* Nees & Arn. ex Munro, *D. asper* (Schult. & Schult. f.) Backer ex K. Heyne, and *D. giganteus* Munro.

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METHODS AND RESULTS

Plant material was obtained from young leaves of 24 individuals of *D. sinicus* from the following two locations (12 individuals each location): Banhong, Cangyuan County (23°17′42″N, 99°05′58″E; straight culmed) and Mengma, Menglian County (22°26′36″N, 99°36′31″E; bending culmed), both of which are located in Yunnan Province, China. Leaf samples from four other bamboo species were collected from the Bamboo Garden of the Southwest Forestry University (SWFU) in Kunming, Yunnan, China. Vouchers were deposited at the Herbarium of SWFU (SWFC) (voucher accession numbers: YangHQ20100011–YangHQ20100034 [*D. sinicus*], Gu201101 [*D. latiflorus*], Gu201102 [*D. hamiltonii*], Gu201103 [*D. asper*], and Gu201104 [*D. giganteus*]).

We developed microsatellite markers according to the Fast Isolation by AFLP of Sequences COntaining Repeats (FIASCO) protocol (Zane et al., 2002). Total genomic DNA was extracted from a single individual using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). Genomic DNA (~250–500 ng) was completely digested with 2.5 U of *Mse*I restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA), and then ligated to an *Mse*I adapter pair (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) with T4 DNA ligase (Fermentas, Burlington, Ontario, Canada) in a 30-μL reaction mixture. A diluted digestion–ligation mixture (1 : 10) was amplified with the adapter-specific primers *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′) (25 μM). Amplified DNA fragments, with a size range of 200–800 bp, were enriched for repeats by magnetic bead selection with a 5′-biotinylated $(AC)_{15}$ and $(AG)_{15}$ probe, respectively. Enriched fragments were amplified again with adapter-specific primers. PCR products were purified using an E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China). The purified DNA fragments were ligated into the pGEM-T Vector (Tiangen Biotech Co. Ltd., Beijing, China), and transformed into DH5α cells (Tiangen). Positive clones were tested by PCR using $(AC)_{10}$/$(AG)_{10}$ and T7/Sp6

TABLE 1. Characteristics of 16 polymorphic microsatellite markers developed in *Dendrocalamus sinicus.*

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	*A*	H_o	H_e	GenBank accession no.
Den006	F: GTCAGGAGGCACAACAAAAT R: GACCTCTGCTTTCGGATAA	$(TC)_8$	224–236	59	2	0.375	0.311	JQ366033
Den007*	F: AAACAAAGCCAGTACTCACA R: CTAACCCTGAGCGTGACCAC	$(AC)_{15}$	265–283	59	3	0.958	0.637	JQ366034
Den008	F: ATGTGCCAAACTAAAATCTG R: GCAAATGAGTGGAAGTTCGT	$(TG)_9$	250–254	52	2	0.625	0.439	JQ366035
Den009	F: GGACACAATTTCTTCACAAC R: GGGAGAGATTGAAGGACCAT	$(AC)_{15}$	261–271	52	2	0.375	0.311	JQ366036
Den020	F: TGTCTTCTGGGCATATTTCT R: TCACTAATCAGAGCAGAATG	$(TG)_8$	271–281	52	2	0.625	0.439	JQ366039
Den033	F: AGGGAGGCTTGGTGCTTAGA R: CTCACTGTAGTCGCATCAAA	$(TA)_7(TG)_8$	270–274	50	2	0.375	0.311	JQ366042
Den034*	F: TATACTGAAGACGGTGCCAA R: TTGTTTACGGCTGTGTTACT	$(AC)_8$	236–242	52	2	0.000	0.479	JQ366043
Den036	F: ATCTAGGTGGTATGAACAAT R: CGTATGTATTTGTGTATCGG	$(TA)_6(TG)_{11}$	214–220	50	2	0.625	0.439	JQ366044
Den049*	F: CACATAAACACGCAAACAAC R: TAGCGTCGGTCACTTACTGT	$(AC)_{10}(AG)_7$	176–178	50	3	1.000	0.630	JQ366047
Den058	F: TGTGCTATTCTGTGTTGATT R: GCTTTCATTTACTGCCCTCT	$(TC)_{13}(AC)_{10}$	105–111	48	2	0.625	0.439	JQ366048
Den067*	F: TACCTTCCCTTTTTCAGCCCAT R: TAGGGAAATAAACCAGTAAATG	$(TG)_{10}$	128–152	50	3	1.000	0.630	JQ366049
Den075*	F: ATTATGTCGGCAAAAAAGCAGA R: CGGTCAATGTGAAGTGTCT	$(TG)_7$	111–123	52	4	1.000	0.750	JQ366050
Den086*	F: GCTACCGATAAGATTTTCCA R: CTATTTTCATCAACATCTACAC	$(TG)_{30}$	163–175	48	4	1.000	0.754	JQ366052
Den091*	F: TGGGACAGAACACTAACAAT R: CCGTTGTTTGTTTTCCCCCCTC	$(AC)_9$	116–122	55	4	1.000	0.622	JQ366053
Den096	F: AGAAGAAGGGAACTCACAAA R: ATGTGTTTTTTCGGGGATTG	$(AC)_7$	158–168	52	2	0.375	0.311	JQ366055
Den103*	F: AACGCACGGGCACTCTACTA R: ACGGTTATTGGTTCTTTGTA	$(TG)_8$	154–182	52	3	1.000	0.630	JQ366057

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; T_a = annealing temperature.
* Indicates loci that deviated from Hardy–Weinberg equilibrium ($P < 0.01$).

as primers, respectively. In total, 355 clones with positive inserts were sequenced with an ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Carlsbad, California, USA). A total of 251 sequences were found to contain microsatellite repeats, and 115 of them were suitable for designing locus-specific primers, using Oligo 7.0 software (Rychlik, 2007).

Polymorphisms of all microsatellite loci were assessed in 24 individuals of *D. sinicus*. PCR reactions were performed in 15-μL reactions containing 30–50 ng of genomic DNA, 0.6 μM of each primer, 7.5 μL 2× *Taq* PCR MasterMix (0.1 U *Taq* polymerase/μL, 0.5 mM dNTP each, 20 mM Tris-HCl [pH 8.3], 100 mM KCl, 3 mM $MgCl_2$; Tiangen). PCR amplifications were conducted under the following conditions: 95°C for 3 min followed by 30–36 cycles at 94°C for 30 s, at the annealing temperature for each specific primer (optimized for each locus, Table 1) for 30 s, at 72°C for 1 min, and a final extension step at 72°C for 7 min. PCR products were sized on the QIAxcel Capillary Electrophoresis System (QIAGEN, Irvine, California, USA) using an internal 25–450-bp size standard. Raw allele length data were converted into allelic classes by the statistical binning of the alleles into base pair (bp) size categories using the program FLEXIBIN (Amos et al., 2007). Because of a potential error of up to 5 bp when scoring alleles on a QIAxcel Capillary Electrophoresis System, we used ±3 bp as allelic category size determination standard error range in FLEXIBIN. The scores were also checked visually. These allelic classes were used for determining the number of alleles per locus in all subsequent analyses.

Of 115 primer pairs, 30 successfully amplified in all samples and 16 primer pairs displayed polymorphism (Table 1). The genetic statistics were calculated using the package GENEPOP (version 4.0; Raymond and Rousset, 1995), including the number of alleles per locus (*A*), observed heterozygosity (H_o), and expected heterozygosity (H_e). The same program was used to test Hardy–Weinberg equilibrium (HWE). *A* ranged from two to four, with a mean of 2.6. H_o and H_e varied from 0 to 1.000 and from 0.311 to 0.754, respectively. Eight loci deviated from HWE ($P < 0.01$, Table 1). Except Den091, the remaining 15 locus pairs were linked with one another ($P < 0.01$). Some studies also indicated that populations of clonal plants may show significant multilocus linkage disequilibria (e.g., Kjølner et al., 2006). Woody bamboos are typical clonal plants, and these results probably reflect this character of clonal plants. The transferability of the 16 primer pairs was tested on four *Dendrocalamus* species, and 11 (68.8%), 13 (81.3%), 12 (75.0%), and nine (56.3%) primer pairs were successfully amplified in *D. asper*, *D. giganteus*, *D. hamiltonii*, and *D. latiflorus*, respectively (Table 2).

TABLE 2. Cross-species amplification of *Dendrocalamus sinicus* microsatellite primers in other *Dendrocalamus* species.

Species	Den006	Den007	Den008	Den009	Den020	Den033	Den034	Den036	Den049	Den058	Den067	Den075	Den086	Den091	Den096	Den103
D. asper	+	+	−	−	+	+	+	−	+	+	+	+	+	−	+	−
D. giganteus	+	+	−	−	+	+	+	+	+	+	+	+	+	−	+	+
D. hamiltonii	+	+	−	−	+	+	+	−	+	+	+	+	+	−	+	+
D. latiflorus	+	+	−	−	+	+	−	−	+	−	+	+	+	−	+	−

Note: + = successful amplification; − = failed amplification.

CONCLUSIONS

A total of 16 polymorphic microsatellite loci have been identified and characterized for *D. sinicus*. They will be used to assess genetic variation in this species, which will be helpful for its conservation in the future. The potential applications of these novel markers include provenance identification of *D. sinicus*, especially if some markers reveal site-specific loci in further study.

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