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TECHNICAL NOTE

Development of microsatellite markers for *Bambusa arnhemica* (Poaceae: Bambuseae), a bamboo endemic to northern Australia

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Abstract *Bambusa arnhemica* is a bamboo species endemic to northern Australia. We isolated and characterized nine microsatellite loci from this species. The number of alleles ranged from 2 to 16 with an average of 6.8, and expected heterozygosities from 0.40 to 0.84 with an average of 0.69. The markers described here will be useful to investigate clump structure, evolution of the bamboo flowering wave, patterns of gene flow, and the biogeographic history of *B. arnhemica* in Australia.

Keywords Bamboo · *Bambusa arnhemica* · Microsatellite

Bambusa arnhemica (Poaceae: Bambuseae) is a 10–20 m tall, pachymorph (clumping) bamboo endemic to the north-west of the Northern Territory, Australia. After 40–50 years of vegetative development, *B. arnhemica* flowers gregariously in a complex spatio-temporal wave, and then dies (Franklin 2004). As with many other bamboos, the timing of flowering is believed to be under strong genetic control (Franklin 2004). In order to test hypotheses about the evolution of flowering behaviour in *B. arnhemica*, we developed nine microsatellite markers for this species.

Microsatellites were developed using the improved technique for isolating codominant compound microsatellite markers of Lian and Hougetsu (2002) and Lian et al. (2006). An adaptor-ligated, restricted DNA library for *B. arnhemica* was constructed according to the following procedure: genomic DNA was extracted from fresh leaves using a DNeasy Plant Mini Kit (Qiagen) and digested with the blunt-end restriction enzyme *Eco*RV. The restriction fragments were then ligated with a specific blunt adaptor (consisting of the 48-mer: 5′-GTAATACGACTCACT ATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3′ and an 8-mer with the 3′-end capped with an amino residue: 5′-ACCAGCCC-NH_2-3′) using the Takara DNA ligation kit (Takara). To block polymerase-catalysed extension of the 8-mer adaptor strand, the ligated fragments were further treated with ddGTP by AmpliTaq Gold (Applied Biosystems). Fragments were amplified by PCR from the *Eco*RV DNA library using compound SSR primer $(AC)_6(AG)_5$ or $(TC)_6(AC)_5$ and an adaptor primer (5′-CTATAGGGCACGCGTGGT-3′). The amplified fragments, ranging from 400 to 800 bp, were then separated on a 1.5% LO3 agarose gel (Takara) and purified using the QIAquick Gel Extraction Kit (Qiagen). The purified DNA fragments were cloned using the QIAGEN PCR Cloning plus Kit (Qiagen) following the manufacturer's instructions. The cloned fragments were amplified using the M13 forward and reverse primers from the plasmid DNA. Amplified fragments were sequenced using the BigDye

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Terminator Cycle Sequencing Kit (Applied Biosystems). For each fragment containing a compound SSR sequence at one end, a specific primer was designed from the sequence flanking the compound SSR using OLIGO software (National Biosciences). Polymerase chain reaction amplifications were performed following the standard protocol of the Qiagen Multiplex PCR Kit (Qiagen) in a final volume of 10 μl, which contained 5 ng of extracted DNA, 5 μl of 2× Multiplex PCR Master Mix, and 0.2 μM of each multiplexed primer. Compound SSR primers ($(AC)_6(AG)_5$ or $(TC)_6(AC)_5$) were labeled with fluorochromes 6-FAM or VIC (Applied Biosystems). Polymerase chain reaction amplifications were performed with the GeneAmp PCR System 9600 thermal cycler (Applied Biosystems) using the following conditions: initial denaturation at 95°C for 15 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing for each of the designed specific primers at the temperatures shown in Table 1 for 1 min 30 s, extension at 72°C for 1 min, and final extension at 60°C for 30 min. The size of the PCR products was measured using

Table 1 Characteristics of nine compound microsatellite loci for *Bambusa arnhemica*

Locus	Repeat motif	Primer sequences(5′–3′)	T_a(°C)	Size range (bp)	Accession no.
BA02	$(AC)_6(AG)_{17}$	ACACACACACACAGAGAGAGAG TGAGAGCTTTGGAGAATG	57	65–79	AB368972
BA05	$(AC)_6(AG)_7$	ACACACACACACAGAGAGAGAG CCGTAATAAACGTGATTG	57	71–77	AB368973
BA11	$(AC)_6(AG)_8$	ACACACACACACAGAGAGAGAG CAGCAAGTAGACACATGATTC	57	209–219	AB293453
BA17	$(AC)_6(AG)_6$	ACACACACACACAGAGAGAGAG CAGATGCAGATTGCTGTACTAT	57	175–181	AB293454
BA18	$(AC)_6(AG)_{10}$	ACACACACACACAGAGAGAGAG CCTCCCTCATCTACCTCTCA	57	80–96	AB293455
BA26	$(TC)_6(AC)_6$	TCTCTCTCTCTCACACACACAC GGTCTTGTTATGTATTAGGG	57	145–147	AB293456
BA29	$(TC)_6(AC)_{13}$	TCTCTCTCTCTCACACACACAC AGAAGCTTACATCACAAGAAA	57	92–104	AB368974
BA41	$(TC)_6(AC)_9$	TCTCTCTCTCTCACACACACAC ACGACCAGAATAGCACACTT	57	255–261	AB293457
BA45	$(TC)_6(AC)_6$	TCTCTCTCTCTCACACACACAC CGAATCTTAAAGCAAATCAAT	57	85–123	AB293458

Ta, annealing temparature of primer pair

Table 2 Variability of nine microsatellite loci in four populations of *B. arnhemica*

	Melville ($n = 24$)			Daly ($n = 26$)			Reynolds ($n = 22$)			Mary ($n = 23$)			Total ($n = 95$)		
	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E
BA02	5	0.63	0.79	4	0.35	0.63	4	0.41	0.48	7	0.74	0.82	8	0.53*	0.83
BA05	3	0.38	0.58	3	0.12	0.11	1	0.00	0.00	3	0.35	0.37	4	0.21*	0.51
BA11	5	0.50	0.51	4	0.42	0.55	2	0.50	0.46	5	0.39	0.50	6	0.45*	0.71
BA17	3	0.33	0.44	3	0.27	0.53	2	0.05	0.05	3	0.30	0.46	4	0.24*	0.68
BA18	6	0.54	0.73	4	0.42	0.45	1	0.00	0.00	3	0.26	0.39	9	0.32*	0.79
BA26	2	0.33	0.45	1	0.00	0.00	1	0.00	0.00	2	0.22	0.32	2	0.14*	0.40
BA29	3	0.63	0.61	4	0.54	0.62	4	0.64	0.69	6	0.52	0.72	8	0.58*	0.74
BA41	2	0.21	0.25	3	0.42	0.47	3	0.50	0.51	3	0.17	0.17	4	0.33*	0.67
BA45	5	0.46	0.48	6	0.62	0.81	6	0.36	0.40	12	0.52	0.75	16	0.49*	0.84
Total	3.8	0.44	0.54	3.6	0.35	0.46	2.7	0.27	0.29	4.9	0.39	0.50	6.8	0.36	0.69

n, number of individuals genotyped; A, number of alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity. A significant deviation from Hardy–Weinberg equilibrium expectations is indicated by *($P < 0.05$)

the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and GENESCAN^{TM} analysis software (Applied Biosystems).

Nine polymorphic loci were identified that showed a clear, strong single band for each allele (Table 1). Polymorphism was evaluated from leaf samples for 95 individuals from four populations of *B. arnhemica* which occurred in different watersheds and flowered in different year (Melville Island population flowered in 2005, Daly River population flowered in 2000, Reynolds River population flowered in 2002, and Mary River population flowered in 1996). The number of alleles per locus ranged from 2 to 16 with an average of 6.8. The observed and expected heterozygosities (H_O and H_E) ranged from 0.14 to 0.58 and from 0.40 to 0.83, with averages of 0.36 and 0.69, respectively (Table 2). Deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium between loci were tested with FSTAT (version 2.9.3; Goudet 1995). Significance levels were adjusted using Bonferroni correction for multiple testing. Although significant deviations ($P < 0.05$) from HWE were observed at all loci over the whole data set ($n = 95$ individuals; Table 2), no significant deviations were observed at the population level (Table 2). These results indicate that level of gene flow among populations is restricted and that random mating occurs within populations. There was no evidence of significant linkage disequilibrium. The microsatellite markers described here will be useful for investigating the genetic structure of clumps, the evolution of gregarious flowering behaviour, patterns of gene flow, and the biogeographic history of *B. arnhemica* in Australia.

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