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Developing SSR primers in *Bombax ceiba* (Bombacaceae)

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Abstract: *Bombax ceiba* is a multipurpose tree species of tropical forests. Nine novel polymorphic microsatellite loci for *B. ceiba* were isolated and characterized through the combined biotin capture method. Markers were tested on 32 individuals collected from dry-hot valleys in southwest China. The number of alleles ranged from 2 to 6. The expected and observed heterozygosity values ranged from 0 to 0.525 and 0.121 to 0.561, respectively. One locus showed significant deviation from Hardy-Weinberg Equilibrium. No significant linkage disequilibrium was found. The microsatellite markers that were characterized in this study were the first to be developed for *B. ceiba* and would have great potential for different genetic studies of wild populations of this species.

Key words: *Bombax ceiba*; microsatellite markers; genetic diversity
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木棉的SSR引物开发

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摘 要: 木棉是分布于热带地区的重要经济树种。通过磁珠富集方法开发了木棉的SSR引物,并在采自于西南干热河谷的32个木棉个体中进行了多态性验证。结果表明:这些位点等位基因数量为2~6个,期望杂合度和观测杂合度范围分别为0~0.525和0.121~0.561。仅有一个位点显示Hardy-Weinberg平衡的偏离。这些微卫星标记的开发在木棉属中尚属首次,对于木棉的野生种群遗传多样性研究和木棉遗传资源利用上有很高的潜在价值。

关键词: 木棉; 微卫星标记; 遗传多样性

Bombax ceiba (Bombacaceae), commonly known as Cotton Tree, is a multipurpose tree species of tropical forests, providing food, fodder, fiber, and medicine besides many ecological benefits (Jain *et al*., 2011). This lofty tree species is found in temperate and tropical Asia, Africa, America and Australia, is an important part of many tropical dry deciduous forest ecosystems (Li, 1987). To better understand the genetic diversity, population structure of *B. ceiba*, microsatellite loci, useful codominant markers for population genetics research and genetic improvement, are needed.

Microsatellites, or tandem simple sequence repeats (SSR), have been found in every organism studied so far since they may be highly polymorphic are useful genetic markers (Tautz & Renz, 1984). To amplify microsatellite loci by PCR, primers must be developed from

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the DNA that flanks specific microsatellite repeats. Although microsatellite loci have now been developed for hundreds of species, these loci have not yet been isolated from many additional species of interest and remain to be developed. This study is the first attempt to isolate and characterize microsatellite markers for this important tree species. Here we describe the isolation and evaluation of 9 novel microsatellite loci in *B. ceiba*, which will be used in further assessment of the genetic diversity and germplasm characterization to facilitate molecular marker-assisted selection and breeding of this species and its relatives.

1 Materials and Method

Genomic DNA for constructing a microsatellite-enriched library was extracted from approximately 30 mg of silica gel-dried, leaf tissue using the modified cetyltrimethylammonium bromide (CTAB) method (Doyle JJ & Doyle JL, 1987). The selective capture of microsatellite loci using magnetic beads was used as a methodological strategy to clone the microsatellite loci. The microsatellite isolation procedure followed the method of Hauswaldt & Glenn (2003). Briefly, about 300 ng genomic DNA was completely digested with a restriction enzyme *Rsa*I (NEB) and then ligated to SuperSNX linkers. DNA fragments were enriched for microsatellite locus by Dynabeads M-280 magnetic streptavidin beads (Invitrogen, Grand Island, New York, USA) selection with 5′-biotinylated (AG) 12, (AT) 8, (CG) 12, (GT) 12, (ACG) 12 and (CCA) 8 probes. Captured fragments were reamplified with adaptor-specific primers by following the program: 95 ℃ for 2 min, 25 cycles of 95 ℃ for 20 s, 60 ℃ for 20 s, 72 ℃ for 90 s followed by an elongation step of 30 min at 72 ℃. Polymerase chain reaction (PCR) products were linked into the pGEM-T Easy Vector (Promega, USA) and transformed into JM109 cells. Positive clones were selected by blue and white screening and tested by PCR using two primers M13 +/M13 −. PCR products (> 350 bp) were sequenced on an ABI 3730xl Genetic Analyzer (Applied Biosystems) using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). The sequences containing motifs repeating more than three times were regarded as microsatellites.

After discarding sequences with few repeat regions or not suitable for designing primers, a total of 39 clones were found out of the sequenced 210 sequences to possess microsatellite motifs using the program SSR Hunter 1.3.0 (Qiang Li, Nangjing Agricultural University, Nanjing, China). These sequences were used the program Primer Premier version 5.0 (Clarke & Gorley, 2001) to design primers.

Three individuals from each population were selected to evaluate the optimal annealing temperature. A gradient PCR procedure was performed using an initial step of 94 ℃ for 4 min; followed by 35 cycles of 94 ℃ for 40 s, 48-65 ℃ for 50 s, and 72 ℃ for 60 s; and a final extension of 72 ℃ for 10 min. PCR was performed in a 25 μL mixture containing 40 ng of genomic DNA, 0.3 μL dNTPs (10 mmol/L), 0.3 μmol/L of each primer, 2 μL of 10 ×PCR buffer, and 0.6 U of Taq polymerase (TaKaRa Biotechnology Co.). In order to analyze the genetic polymorphism of these isolated microsatellite loci, 32 individuals were selected from dry-hot valleys in southwest China for genotyping. The microsatellite loci were amplified by PCR using the same procedure for the evaluation of the optimal annealing temperature. PCR productions were separated using PAGE electrophoresis using a 50-bp DNA Step Ladder to determine the allele size.

2 Results

A total of 19 out of the 39 primers pairs successfully amplified the target regions and 9 of them showed polymorphic banding patterns (Table 1). The characteristics of the nine novel polymorphic microsatellite loci for *B. ceiba* are listed in Table 1 and their corresponding sequences are deposited in Genbank under the accession numbers from KC471333 to KC471341. The number of alleles per locus (*Na*), observed heterozygosity (*Ho*) and expected heterozygosity (*He*) were calculated using software GenALEx 6.4 (Peakall & Smouse, 2006); nine primer pairs were successfully amplified, and all of them showed

polymorphism. The observed and expected heterozygosities varied from 0 to 0.525 and 0.121 to 0.561, respectively (Table 1).

Table 1 Characteristics of nine polymorphic microsatellite markers from *Bombax ceiba*

Locus	Primer sequence (5′→3′)	Repeat motif	Size range (bp)	*Ta* (℃)	GenBank Accession No.	*Na*	*Ho*	*He*	P_{HW}
BC01	F: CCATCAGCGAGGACATTG R: CATAAGGCATCGGCATAG	$(GGA)_4AGTGA(TGG)_5$	370-382	53	KC471333	4	0.121	0.459	0.372
BC02	F: AAGGCTACTACAACTTGACTG R: CTTTGCTCTATTCCCACA	$(ATG)_3A(TGG)_3$	167-170	52	KC471334	2	0.000	0.169	0.521
BC03	F: GATGCTTCTCCTGCTTTA R: AATGTGGGCTTATTGTCT	(GAC)6	334-340	50	KC471335	3	0.172	0.205	0.339
BC04	F: ATTTGAATCTTGCGTGTTAC R: TTCCTCACCCTCCTCTTT	(TG)6(TGCG)3(TG)4	321-335	52	KC471336	6	0.467	0.561	0.441
BC05	F: TGGTGGTAAAGCAAGGATCG R: TGCACTGAGTGACCATGACA	(TG)12	103-117	59	KC471337	5	0.525	0.533	0.213
BC06	F: CTTGAGAGCTCCGCTTGAAC R: AACGGGAATGGGAAAAGTG	(CCA)5	167-173	52	KC471338	3	0.210	0.321	0.002*
BC07	F: AAAGCCAACATGATCGGAGAT R: TGGCAGTTTCGGCATACA	(GTG)9	92-107	59	KC471339	5	0.456	0.452	0.078
BC08	F: AACGGGAATGGGAAAAGTG R: CTTGAGAGCTCCGCTTGAAC	(GGT)6	136-148	50	KC471340	4	0.328	0.532	0.568
BC09	F: AGCTTTACAAGCCTCATG R: AAGGTATCTATTCCAGCA	(GT)9	290-298	50	KC471341	5	0.378	0.541	0.662

Ta: annealing temperature (℃); *Na*: number of alleles; *Ho*: observed heterozygosity; *He*: expected heterozygosity; P_{HW} from exact tests for Hardy-Weinberg equilibrium(* $P<0.01$).

Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were calculated using ARLEQUIN 3.5 (Excoffier & Lischer, 2010). After Bonferroni adjustments, one locus, BC06, was found to be deviated from HWE ($P<0.01$) (Table 1), and no significant evidence of linkage disequilibrium was observed.

3 Conclusions

The number of loci scored, degree of polymorphism of each locus and sample size are of paramount importance for the statistical power of microsatellites to be effective (Zane *et al*., 2002). In this study, 9 novel polymorphic microsatellite markers were characterized out of a total of 210 clones sequenced. These primers are the first to be developed for *B. ceiba* and have great potential for use in different genetic studies of wild and cultivated populations of this species. These markers will be used to gain a better understanding of various evolutionary questions including population genetic diversity and differentiation, population demography, and gene flow of *B. ceiba* are also expected to be useful for construction of linkage maps and marker assisted selections of this useful species.

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