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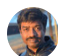

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Identification of novel microsatellite markers for *Saraca asoca*, a medicinally important tree species in India

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Introduction

Saraca asoca (Roxb.) Wilde (Caesalpiniaceae) is a medicinally important and globally vulnerable plant species found in the evergreen forests of India (Thakur *et al.* 1989). *S. asoca*, commonly known as Ashoka tree, is considered as one of the sacred trees of India and is highly prized for its beautiful foliage and fragrant flowers. Almost all parts of the tree are known to have important medicinal properties including antiviral (Hattori *et al.* 1995), oxytotic (Satyavati *et al.* 1970), menorrhagic, anti-HIV (Kusumoto *et al.* 1995) and antibacterial activities (Annapurna *et al.* 1999). The flower extract is commonly used in diabetes and cancer treatments (Anonymous 1952; Mukherji *et al.* 1970; Verghese *et al.* 1992). Overharvesting of *S. asoca* due to its high medicinal value along with high deforestation rates, habitat fragmentation and illegal encroachments of its natural habitats have resulted in severe reduction in natural populations of this species (Gowda *et al.* 2002). This species is currently listed as a 'globally vulnerable' species by the IUCN (http://www.iucnredlist.org/apps/redlist/details/34623/0). In this paper, we report identification of novel microsatellite markers and discuss the utility of these markers in addressing questions related to the population genetics of this species. Ten microsatellite markers were identified in this species. These microsatellite loci have 2–22 alleles per locus; with observed and expected heterozygosity of 0.001–1.00 and 0.273–0.964, respectively. These markers will be invaluable in gaining insights into the population genetics of the species for formulating sound conservation and management strategies.

Material and methods

The leaf samples from *S. asoca* (Roxb.) trees in the Western Ghats of India were collected and genomic DNA was extracted using the CTAB method (Doyle and Doyle 1987). The extracted DNA was purified, dissolved in Tris-EDTA buffer and used for microsatellite identification through the enrichment-subtractive hybridization protocol with minor modifications (Glenn and Schable 2005). Genomic DNA was digested with *Rsa*I (New England Biolabs, Ipswich, Massachusetts, USA) and ligated to linker oligonucleotides SNX-F (5′-GTTTAAGGCCTAGCTAGCAGAATC) and SNX-R (5′-ATTCTGCTAGCTAGGCCTTAAACAAAA) using the Rapid DNA Ligation kit (Fermentas International, Hanover, Maryland, USA). Biotinylated oligonucleotides of selected repeats (microsatellite probes) were used for hybridizing with the DNA fragments and magnetically captured using Dynabeads (Sigma-Aldrich, Bangalore, India). The captured DNA was washed, amplified and cloned to pTZ57R/T plasmid vector using Fermentas TA Cloning Kit. The positive colonies (white) were picked and subjected

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to PCR using the universal M13 forward and M13 reverse primers. The amplified PCR products were sequenced using Cycle sequencer (ABI PRISM 3100 Genetic Analyzer) (Applied Biosystems, Chromous Biotech, Bangalore, India). Sequences that contained microsatellites were short-listed and the vector and linker sequences were edited. The primers were designed for the flanking sequences of the microsatellites by using web-based software Primer3 (Rozen and Skaletsky 2000). Out of the primers suggested by the program, primers of about 20 bp length with annealing temperatures close to 60°C were chosen for primer synthesis.

A total of 15 microsatellite primer pairs were synthesized and screened for polymorphism. Amplification was carried out in an Eppendorf Thermo Cycler, containing reaction mix of 20–30 ng of DNA, 1 mM of dNTP, 5 pmol of each primer, 1× buffer, *Taq* DNA polymerase 0.2–0.3 U (Chromous Biotech, Bangalore, India). PCR conditions for all loci were 94°C for 3 min, 35 cycles of 94°C for 1 min, annealing temperature for 1 min and 72°C for 10 min. A final elongation step at 72°C for 10 min was carried out.

After initial screening of the 15 primers, 10 primer pairs that showed polymorphism were retained for further analysis. Six of these loci were dinucleotide repeats, one each were mononucleotide and trinucleotide repeats and two were penta nucleotide repeat (table 1). The 5′ end of the forward primer of six loci were labelled with fluorescent dyes (SAR4 and SAR7-FAM, SAR5-NED, SAR10-PET and SAR6-VIC), and individuals were genotyped using ABI PRISM 3130 Genetic analyser (Applied Biosystems, Chromous Biotech, Bangalore, India). The electropherograms were analysed using Gene Scan 3.7 and Genotyper 3.7 software packages (Applied Biosystems, Chromous Biotech, Bangalore, India). The genotyping of samples for the remaining four primers were performed using polyacrylamide gel electrophoresis. The aliquots of 5 μL of PCR products were electrophoresed along with a 20 bp ladder on 12% polyacrylamide gel. The gel was run at 100 V for about 12–13 h. After electrophoresis, the gels were silver stained as described by Creste *et al.* (2001) for visualization of DNA fragments (figure 1).

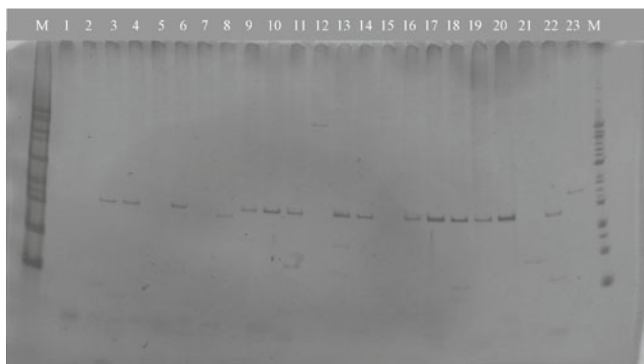

Figure 1. Polyacrylamide gel electrophoresis showing polymorphism using the primer SAR13 (M, 20 bp ladder).

Table 1. Polymorphism data for 10 microsatellites in *Saraca asoca*.

Locus and GenBank accession number	Repeat unit	Forward primer sequence 5′–3′	Reverse primer sequence 5′–3′	Allele size range (bp)	T_a (°C)	*n*	A	H_O	H_E	HWE (*P*)
SAR1 JQ406590	$(CT)_8$	AGAATCCCTTCTTCTTCC	AATCACTCATCAGGTCCAAC	380–410	56	20	05	0.90	0.71	0.0254
SAR4 JQ406593	$(TGG)_6$	AGGGGAAAGAATTTTACCTG	ATGATTACGCCAAGCTCTAA	176–220	35	23	17	0.60	0.88	0.0024
SAR5 JQ406594	$(CT)_8$	AAAGTAATAGATCCCGCACA	GATCCCAGAAACGATGAGTA	200–219	55	23	10	0.56	0.76	0.0001
SAR6 JQ406595	$(AG)_{15}$	TTGATCATCTCATTTCCCTTA	GACTTGGTTCTTAGCAGTGG	251–298	55	23	20	0.74	0.93	0.0001
SAR7 JQ406596	$(AAAAG)_4$	GGAAAAGTAGTGGCAGAGA	ATGATTACGCCAAGCTCTAA	301–397	43	22	20	0.75	0.96	0.0001
SAR8 JQ406597	$(T)_{19}$	TGTTGAGATGATGGATGTTG	CGAATCCCTTTGAATTTTT	310–350	43	22	05	0.95	0.78	0.0464
SAR9 JQ406598	$(TC)_{19}TA(TC)_8$	AAGGCTTGTCTCACATCACT	CAATTTCACACACGAAACAC	350–390	50	22	05	0.68	0.69	0.3862*
SAR10 JQ406599	$(CT)_{10}TT(CA)_7$	CAGACCTCTTGATTCTTGCT	CGACTTCGAAGCTTGTTACT	305–340	55	23	19	1.00	0.90	0.0006
SAR13 JQ406601	$(AAAGG)_5$	GAATCATTTCGATGGGGTTG	TGCGACGCTAATAATCATGG	300–320	59	19	02	0.001	0.27	0.0003
SAR3 JQ406592	$(CT)_7CATC(CT)_3$	AGGATCCATCCCCTCTCT	GAGAGGATGAGAGACACCTG	140–200	45	22	06	1.0	0.72	0.0436

T_a, annealing temperature; *n*, number of individuals; A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, Hardy–Weinberg equilibrium.
* Indicates loci that showed deviation from HWE.

Results and discussion

Nineteen to twenty-three individuals of *S. asoca* were genotyped at 10 loci to assess the genetic variability. The number of alleles, allele range, and expected and observed heterozygosity (H_E and H_O) at each microsatellite loci were calculated using Arlequin ver. 3.0 (Excoffier *et al.* 2005) and are presented in table 1. The number of alleles per locus ranged from 2 to 20. The observed and expected heterozygosity ranged from 0.001 to 1.00 and 0.273 to 0.964, respectively. The tests for departure from Hardy–Weinberg equilibrium (HWE) were performed using Markov-chain random walk algorithm (Guo and Thompson 1992) using the program Arlequin 3.1 (table 1). Only one loci (SAR9) showed deviation from HWE ($P < 0.05$), perhaps either due to skewed sex ratio or because of the breeding system. The exact test for genotypic linkage disequilibrium between loci based on 4500 permutations using the FSTAT ver. 2.9.3 (Goudet 1995) was performed and none of the loci showed significant linkage disequilibrium after multiple tests correction of the probability value ($P > 0.0011$). The microsatellite markers reported in this paper are invaluable for researchers to study population genetic structure of the species to formulate sound conservation and management programmes.

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