

PRIMER NOTE

Isolation and characterization of six novel microsatellite markers for mulberry (*Morus indica*)

RAMESH K. AGGARWAL,* D. UDAYKUMAR,* P. S. HENDRE,* A. SARKAR† and LALJ. I. SINGH*

*Centre for Cellular and Molecular Biology, Uppal Road, Tarnaka, Hyderabad 500007, India, †Central Sericultural Research and Training Institute, Srirampura, Mysore 570008, India

Abstract

Genetic characterization of germplasm resources is necessary for their effective management and efficient utilization, especially for species like mulberry in which the available germplasm exhibits rich phenotypic diversity with almost no information about its genetic base. Here we present the first report on the isolation of six novel microsatellite markers of mulberry, developed from an enriched genomic library of *Morus indica*. These markers revealed a high degree of polymorphism (14–26 alleles per locus; polymorphic information content, 0.85–0.90) and a broad cross-species affinity when tested on a set of 43 elite genotypes including 13 related *Morus* species. The data thus demonstrate their utility as potentially efficient genetic markers for germplasm characterization, crop improvement and molecular systematics of mulberry.

Keywords: diversity, genomic library, genotype, microsatellite, *Morus*, mulberry

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The genus *Morus* is crucial to the sericulture industry as mulberry leaves are an exclusive food source for *Bombyx mori* L. There is a great diversity in mulberry species across Asia but information regarding their origin, domestication and genetic relationships is generally lacking. Despite the availability of considerable diversity in the existing gene-pool, mulberry improvement through conventional breeding is severely constrained due to multiple factors, such as lack of genetic markers and suitable/efficient selection strategies, outcrossing behaviour and a long breeding cycle. These constraints call for recourse to newer high genetic resolution approaches, such as those based on DNA markers providing means for efficient germplasm characterization, deciphering population structures, developing genetic linkage maps and thus, acceleration, reliability and directionality to the variety of developmental efforts. In recent times, a plethora of DNA marker approaches has been developed. Among these, it is the short-sequence repeats (SSRs)-based microsatellites that have proven to be the markers of choice for genetic studies due to their codominant nature, abundance and inherent potential for variation, stability, sensitivity and suitability for automation

(Powell *et al.* 1996; Temnykh *et al.* 2001). Despite these apparent advantages, no such microsatellite markers have so far been developed for mulberry. Here we describe the first successful effort to develop new mulberry-specific SSR markers having potential for use as genetic markers.

A small SSR-enriched genomic library of mulberry was prepared using a simplified protocol based on the biotinylated-oligonucleotide capture and polymerase chain reaction (PCR) enrichment methods of Bloor *et al.* (2001) and Edwards *et al.* (1996). Genomic DNA was isolated from leaf samples of *Morus indica* variety S-13 following the method of Aggarwal *et al.* (2002). About 10 µg genomic DNA was digested with *Rsa*I and *Hae*III (NEB) and 0.5–1.5-kb DNA fragments were eluted and ligated with *Mlu* adaptors (Edwards *et al.* 1996). Digested, adaptor-ligated DNA fragments were hybridized to 5' biotinylated oligonucleotides [(GA)₁₅, (CA)₁₅, (AGA)₁₀ and (CAA)₁₀] and SSR-rich hybridized genomic fragments were recovered by capturing with streptavidin-conjugated magnetic beads (Dynabeads; DYNAL). The eluted fragments were amplified using *Mlu* adaptor-specific primers, cloned into pMOS vector (Amersham Biosciences) and transformed into the competent *Escherichia coli* DH5α host cells. The recombinant plasmid clones were individually picked up and maintained in 384-well microtiter culture plates containing 10%

Correspondence: Ramesh K. Aggarwal, PhD. Fax: 00-91-40-27160591/27160311; E-mail: rameshka@ccmb.res.in

Table 1 Primer sequences and characteristics of the six mulberry specific microsatellite markers developed in the study

Marker	Repeat motif	Primer sequence (5'→3')	T_a (°C)	Size range (bp)	N	N_a	H_O	H_E	GenBank Accession no.
MulSTR1	(GTT) ₆ + (GTT) ₄	F: GCCGTGTACCAGTGGAGTTTGCA R: TGACCGTTTCTTCCACTTTACCTAATG	55	181–209	43	14	0.93	0.85	AY326440
MulSTR2	(GTT) ₁₁	F: CGTGGGGCTTAGGCTGAGTAGAGG R: CACCACCCTACTTCTCTCTTCCAG	55	163–206	43	16	0.72	0.89	AY326441
MulSTR3	(GA) ₃₃	F: GGGTTGGGTAGATGGGCTTATGTTA R: CCTAATTAACTTTTGGTCACCTCTA	55	107–221	43	26	0.37	0.90	AY326442
MulSTR4	(GAA) ₆	F: GGTCAAGCGCTCCAGAGAAAAG R: GGTGCAGAGGATGAAAGATGAGGT	68	112–146	43	18	0.40	0.89	AY326443
MulSTR5	(CCA) ₈	F: CCCCTGCAATGCCCTCTTTC R: TGGCGAGGCAGGGAAGATTC	68	134–166	43	20	0.91	0.87	AY326444
MulSTR6	(GT) ₁₅	F: TCCTTAGGTTTTTGGGGTCTGTTTACAT R: CCTCATCTCTTTCACCTTATTTGTTG	58	119–181	43	18	0.19	0.85	AY326445

F, Forward primer; R, reverse primer; T_a , polymerase chain reaction annealing temperature; N , number of mulberry genotypes tested for allelic polymorphism; N_a , number of observed microsatellite alleles; H_O , observed heterozygosity; H_E , expected heterozygosity (polymorphic information content).

glycerol-Luria-Bertani medium. About 100 randomly selected clones were used to prepare plasmids by the alkaline lysis method (Sambrook *et al.* 1989) and their inserts were amplified with M13 universal primers. Seventy-two robustly amplified inserts were then sequenced for both strands on an ABI-PRISM 3700 automated DNA sequencer (Applied Biosystems). Fifty-seven of the sequenced clones contained SSR repeats of varying length indicating ~79% enrichment. The repeat regions were identified manually and PCR primers were designed for 15 sequences containing > 18 bp long repeat regions using the GENETOOL program (www.doubletwist.com). The primers were synthesized on a DNA/RNA Synthesizer-394 (Applied Biosystems) with the forward primer of each pair being labelled with 5' fluorescence tag, 6-FAM or HEX.

Initially, all the primer pairs were tested for PCR amplification and then only the six working pairs were used to ascertain their utility as potential genetic markers using a panel of 43 mulberry samples that included species representatives and improved cultivated varieties. The PCR amplifications were carried out in 15- μ L reactions containing 10 ng template DNA, 1 pmol of each primer, 2 mM of MgCl₂, 100 μ M of dNTPs, 1 \times PCR buffer II and 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems) on a Thermocycler-2700 (Perkin-Elmer) programmed to the following cycling profile: initial denaturation at 94 °C for 10 min followed by 35 cycles of 94 °C for 1 min denaturation, primer-specific annealing temperature (Table 1) for 1 min and 72 °C for 1.5 min extension followed by the final extension step of 72 °C for 5 min. Amplified products were resolved through GENESCAN analysis on an ABI-PRISM 377 DNA sequencer, as per the manufacturer's instructions (Applied Biosystems). Resolved microsatellite alleles were

precisely sized using the software GENOTYPER 2.1 (Applied Biosystems) to calculate their number, range and distribution.

The variability of the six microsatellite loci across 43 genotypes is summarized in Table 1. The number of alleles per locus ranged from 14 to 26 with a mean of 18.6. The expected heterozygosity varied from 0.85 to 0.90 whereas the observed heterozygosity ranged from 0.19 to 0.93 with an average of 0.59 (Table 1). Allelic diversity data for the six microsatellite markers were tested for Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) for 19 of the genotypes that belonged to one species i.e. *M. indica*. The HWE and LD tests were done using the Markov chain algorithm and Fisher's exact test in ARLEQUIN version 2.0 (Schneider *et al.* 2000) and also GENEPOP version 3.3 (<http://wbiomed.curtin.edu.au/genepop/>). Analysis revealed significant ($P < 0.05$) deviations from HWE at all of the six microsatellite loci and significant LD for two pairs of markers (MulSTR1/MulSTR2 and MulSTR2/MulSTR3) using ARLEQUIN and four pairs (MulSTR1/MulSTR2, MulSTR2/MulSTR3, MulSTR1/MulSTR5 and MulSTR2/MulSTR5) with GENEPOP. These results were rather as expected as the analysed samples did not represent an interbreeding population but comprised only diverse genotypes (elite, mostly unrelated varieties), which are more akin to a structured population. Analysis to test the cross-species affinities revealed robust amplifications in 13 related *Morus* species (Table 2) suggesting wider potential of the new markers in genetic analysis of mulberry resources.

The present study highlights the utility of the microsatellite markers in mulberry for genotype characterization which, until now, has only been attempted using anonymous, dominant DNA markers like RAPD (Bhattacharya and Ranade 2001) and amplified fragment length polymorphisms

Table 2 Cross-species amplification status of microsatellite markers tested using 14 *Morus* species available in India (in each case one to two representative samples were used for analysis except *Morus indica* for which 18 genotypes were tested)

Marker	<i>M. indica</i>	<i>M. alba</i>	<i>M. multicaulis</i>	<i>M. latifolia</i>	<i>M. sinensis</i>	<i>M. bombysis</i>	<i>M. kagayamae</i>	<i>M. nigra</i>	<i>M. ihou</i>	<i>M. australis</i>	<i>M. laevigata</i>	<i>M. serrata</i>	<i>M. cathayana</i>	<i>Broussonetia</i> sp.
MulSTR1	185–209	181–203	180–203	191–205	181–205	191–205	190–205	191–203	183–204	191–203	186–205	191–203	181–203	205
MulSTR2	166–188	163–206	163–188	166–189	166–188	178–188	184	188	184	172–185	172–188	178–188	184–206	184
MulSTR3	107–156	112–160	107–155	143–178	146–155	142–178	146–221	119–143	122	143–155	112–155	107–122	122	146
MulSTR4	121–134	124	124–128	124–128	128–134	117–131	124	112–124	124	117–124	112–146	121–143	121	124
MulSTR5	138–166	144–166	142–166	144–166	134–166	144–166	160	160	141–162	154–160	134–160	142–160	154–160	166
MulSTR6	132–181	132–146	142–163	142–146	142	142–157	142	130	146	142	135–146	119–130	146	134–146

(Sharma *et al.* 2000). The polymorphic microsatellite markers reported here provide robust, high resolution tools for the efficient genetic assessment of the available mulberry genepool for its conservation, management and gainful utilization. They are also expected to be useful for efficient genetic studies, e.g. linkage analysis, construction of molecular linkage maps and marker-assisted breeding.

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