**Journal of Genetics** Vol. 97, Online Resources, pp. e31–e37
https://doi.org/10.1007/s12041-018-0900-5



**ONLINE RESOURCES**

# A new set of mulberry-specific SSR markers for application in cultivar identification and DUS testing

MARIAN VINCENT PINTO[1], H. S. POORNIMA[1], V. SIVAPRASAD[1] and V. GIRISH NAIK[1,2]*

[1] *Molecular Biology Laboratory-1, Central Sericultural Research and Training Institute, Srirampura, Manandavadi Road, Mysuru 570 008, India*
[2] *Regional Sericultural Research Station, Nanjangud Road, Chamarajanagar 571 313, India*
*For correspondence. E-mail: vgirishnaik@gmail.com.

Received 30 June 2017; accepted 18 July 2017

**Keywords.** mulberry; simple sequence repeats; genotyping; genetic analysis; cultivar identification; DUS testing.

## Introduction

The monophagous domesticated silkworm *Bombyx mori* derives all its nutritional requirement from the mulberry (*Morus* spp.) leaves. Therefore, mulberry has a key role in sustainably improving the quality and yield of silk. Lack of codominant markers for use in genetic mapping has constrained the efforts in marker-assisted breeding for targeted trait improvement. Currently, a total of 443 (160 genomic and 283 genic) mulberry-specific simple sequence repeat (SSR) markers are available for genome analysis (Aggarwal *et al.* 2004; Tani *et al.* 2005; Zhao *et al.* 2005; Gao 2013; Mathithumilan *et al.* 2013; Thumilan *et al.* 2016). The draft genome sequence of *Morus notabilis* was made available to the mulberry research community by He *et al.* (2013). Krishnan *et al.* (2014a) mined SSRs available in the genome sequence and archived them in the MulSatDB. In this study, we report the development and validation of 24 primer pairs for the SSRs archived in MulSatDB. We have used these markers to characterize a set of 216 diverse mulberry germplasm and explore the possibility of using them in cultivar identification.

VGN and MVP conceived the study. HSP and VGN designed the primers. MVP and HSP optimized the PCR conditions. MVP carried out the genotyping and data analysis. MVP and VGN wrote the manuscript. VS coordinated the study. All authors have read and approved the final manuscript.

## Materials and methods

We chose 37 genomic sequences that had a high number of simple di, tri and tetra SSR motifs from the MulSatDB (table 1). Primers for amplification of the SSR regions were designed using the tool WebSat (Martins *et al.* 2009). OligoCalc ver. 3.27 (Kibbe 2007) was used to calculate the $T_M$, length, GC content and to rule-out self-complementarity in the designed primers.

A panel of diverse mulberry germplasm was sampled from the whole germplasm collection so as to retain all the phenotypic and allelic diversity available in the gene bank (Krishnan 2014). A total of 216 accessions from the panel were utilized in the present study. These accessions were of mixed ploidy level (diploids, triploids and tetraploids) and represented 10 mulberry species and their hybrids. Young-leaf material was collected from the *ex situ* gene bank at Central Sericultural Germplasm Resources Centre, Hosur and frozen at –80°C. High molecular weight genomic DNA was extracted from the frozen leaves using HiPurA Plant Genomic DNA Miniprep Purification Kit (HiMedia, Mumbai, India) following the manufacturer's instructions. A small aliquot of the DNA sample was run on 1% agarose gel in 1× TAE at 4 V/cm for 1 h (Sambrook and Russell 2001). The DNA was quantified using NanoDrop 2000C (Thermo Fisher Scientific, Wilmington, USA). DNA samples that appeared as a single, sharp high molecular weight band on the agarose gel with an $A_{260}/A_{280}$ ratio of 1.8–2 were diluted to 5 ng/$\mu$L with $T_{10}E_1$ buffer (pH 8.0) and used in PCR.

Published online: 13 April 2018

**Table 1.** MulSatG: a new series of mulberry-specific SSR markers.

| Marker name | Repeat motif | Primer sequence (5′ → 3′) | $T_M$ (°C) | Length (bases) | GC% | Product size (bp) | NCBI ProbeDB acc. no. |
|---|---|---|---|---|---|---|---|
| MulSatG16983 | $(CT)_{36}$ | F: CCCTTTTCCCTCTCCTCATATC | 60.3 | 22 | 50 | 130 | Pr032825885 |
| | | R: TATGTTTGTGAGTGCATTTGGG | 60.8 | 22 | 41 | | |
| MulSatG149732 | $(TC)_{35}$ | F: CCAACAGCTTCTTTGCTCCTAT | 60.1 | 22 | 45 | 171 | Pr032825881 |
| | | R: GAAAATTGCCCAAGTGAAAGAG | 60.1 | 22 | 41 | | |
| MulSatG140006 | $(AG)_{34}$ | F: GACTCCGTCACAAGGCGTA | 59.8 | 19 | 58 | 269 | Pr032825879 |
| | | R: CTTTGCAGTTTGATGTGGAGAG | 59.9 | 22 | 45 | | |
| MulSatG95145 | $(AG)_{29}$ | F: GTGATGAGAACCTGAAGCTGTG | 59.9 | 22 | 50 | 243 | Pr032825904 |
| | | R: CCAAAAGATGTATGTCCCTGGT | 60.1 | 22 | 45 | | |
| MulSatG110731 | $(CT)_{28}$ | F: GCCCACTTGGACATTAGTCATT | 60.2 | 22 | 45 | 231 | Pr032825872 |
| | | R: TATGGTGATATGGATGGATGGA | 59.9 | 22 | 41 | | |
| MulSatG123204 | $(AG)_{28}$ | F: CCTCCTCATAAGCATCACCATT | 60.3 | 22 | 45 | 258 | Pr032825875 |
| | | R: TAGACATTGGAACAACGCATTC | 60.0 | 22 | 41 | | |
| MulSatG86682 | $(CT)_{26}$ | F: TAGAGTTATGGGCACTTTTGGG | 60.4 | 22 | 45 | 281 | Pr032825900 |
| | | R: TAAGTTTTATTGATGGGTCGCC | 60.2 | 22 | 41 | | |
| MulSatG155763 | $(TC)_{25}$ | F: TTAGGCTTCCAAACTCTCTCCA | 60.4 | 22 | 41 | 183 | Pr032825883 |
| | | R: GCCCAACTTACTCCCTCTCTCT | 60.3 | 22 | 55 | | |
| MulSatG107923 | $(TC)_{24}$ | F: TCCCACTTTTCTCTTCCGTAAT | 59.1 | 22 | 41 | 159 | Pr032825871 |
| | | R: TGCCTGACCCCTCTGAAC | 59.7 | 18 | 61 | | |
| MulSatG118804 | $(AG)_{23}$ | F: ACTACGAATATGACCCAATGACTTC | 59.7 | 25 | 40 | 258 | Pr032825873 |
| | | R: ATGACTGCCCAGAAGATAAGGA | 60.1 | 22 | 45 | | |
| MulSatG143363 | $(ATA)_{17}$ | F: GAGCCTACTTCTTCCCACTTGA | 59.9 | 22 | 50 | 292 | Pr032825880 |
| | | R: TACAACGCGATAACTTCCCTTT | 60.0 | 22 | 41 | | |
| MulSatG94574 | $(AAC)_{15}$ | F: CAACCCCTCTAATCTCAACGAC | 60.0 | 22 | 50 | 236 | Pr032825903 |
| | | R: CAACAATGGAGCAACTCAAAAC | 59.7 | 22 | 41 | | |
| MulSatG45381 | $(AAT)_{13}$ | F: ACTATCGCTCGTCCAGGTGT | 59.8 | 20 | 55 | 266 | Pr032825891 |
| | | R: CTACTCATGCTATGGATGTGGG | 59.5 | 22 | 50 | | |
| MulSatG5166 | $(CTT)_{13}$ | F: TGGTACTTCCTCTCGCGTTAGT | 60.3 | 22 | 50 | 171 | Pr032825893 |
| | | R: CCAAAATATCTCACCTCCAAGC | 60.0 | 22 | 45 | | |
| MulSatG92497 | $(ATT)_{13}$ | F: CTTGGAGGAGAAGAGTGAGGAA | 60.0 | 22 | 50 | 239 | Pr032825902 |
| | | R: AGGAACCACAAAGGAACACAAG | 60.4 | 22 | 45 | | |
| MulSatG100717 | $(TTC)_{11}$ | F: GAATCAAGCAGAAGAAACGTCA | 59.5 | 22 | 41 | 236 | Pr032825869 |
| | | R: GTCACAACAATAGGGACAAGACA | 59.0 | 23 | 43 | | |
| MulSatG107143 | $(AAT)_{11}$ | F: TCATCTCTGTTGGTGTGGTTTT | 59.5 | 22 | 41 | 270 | Pr032825870 |
| | | R: TGCCTCCCTAGTTTCTTCACAT | 60.1 | 22 | 45 | | |
| MulSatG192454 | $(TCT)_{11}$ | F: ACGGTTGTACTTGAGGAACGTC | 60.5 | 22 | 50 | 163 | Pr032825889 |
| | | R: CCTCTTTCTTACTGCTCTCCCA | 60.0 | 22 | 50 | | |
| MulSatG135883 | $(GA)_{23}$ | F: AGAAAAGCGGAGCCGAAAG | 61.9 | 19 | 53 | 202 | Pr032825877 |
| | | R: CCTTCCAATCTCATACTTCCCC | 61.0 | 22 | 50 | | |
| MulSatG12738 | $(AG)_{22}$ | F: GACAAGCCTAAGAAGAAGGGGT | 60.1 | 22 | 50 | 176 | Pr032825876 |
| | | R: TTCTCCAACATGGGTTCCTC | 59.9 | 20 | 50 | | |

**Table 1** (*contd*)

| Marker name | Repeat motif | Primer sequence (5′ → 3′) | $T_M$ (°C) | Length (bases) | GC% | Product size (bp) | NCBI ProbeDB acc. no. |
|---|---|---|---|---|---|---|---|
| MulSatG172234 | $(CT)_{22}$ | F: GACAGTGCTCAACAACCACG | 60.4 | 20 | 55 | 219 | Pr032825886 |
| | | R: GGGGAGGGATTAGAGAGAGAGA | 60.2 | 22 | 55 | | |
| MulSatG75021 | $(GA)_{22}$ | F: CGATATTGGCTAGGAACGG | 58.2 | 19 | 53 | 177 | Pr032825896 |
| | | R: CCCACAGATAAAACCCCATC | 59.1 | 20 | 50 | | |
| MulSatG84275 | $(GA)_{21}$ | F: CATGGAAGACACAGACACAAGC | 60.8 | 22 | 50 | 185 | Pr032825899 |
| | | R: CCAGGTAACAATAACCCCAACC | 61.5 | 22 | 50 | | |
| MulSatG52471 | $(AG)_{20}$ | F: TACAAGGAGAACGGAGACCCTA | 60.1 | 22 | 50 | 168 | Pr032825894 |
| | | R: ACTCTTGGCTATTTCCACGC | 59.3 | 20 | 50 | | |
| MulSatG83251 | $(AG)_{20}$ | F: TCACAGATGTCAAAGCCAGC | 60.0 | 20 | 50 | 180 | Pr032825898 |
| | | R: GTCCACCAAAGTAGCTCCACTC | 60.2 | 22 | 55 | | |
| MulSatG118808 | $(TC)_{19}$ | F: CTTTAACACATGCAGGACTCGG | 61.9 | 22 | 50 | 233 | Pr032825874 |
| | | R: CCATCTCTCTCTTTCTCTCTCCTACC | 61.5 | 26 | 50 | | |
| MulSatG190136 | $(TA)_{19}$ | F: CTCCGAAGAATCCCAGTAGACC | 61.3 | 22 | 55 | 260 | Pr032825888 |
| | | R: CTCCGAAGAATCCCAGTAGACC | 60.7 | 22 | 61 | | |
| MulSatG79725 | $(AG)_{19}$ | F: GAGAACAGTGGGAGTGAACCA | 60.1 | 21 | 52 | 191 | Pr032825897 |
| | | R: CCCCTCTTGGCCTATATCTCTC | 60.4 | 22 | 55 | | |
| MulSatG4597 | $(AG)_{18}$ | F: GTTGCATCCACACAAACCC | 59.8 | 19 | 53 | 280 | Pr032825892 |
| | | R: TGCTCTCTCTCTCTCTCTCTCTC | 59.5 | 25 | 52 | | |
| MulSatG178203 | $(TCT)_{10}$ | F: GAAAATCGTCTTCTGTGCGG | 60.8 | 20 | 50 | 162 | Pr032825887 |
| | | R: GCGGGTTTAGATTCAGGGA | 60.0 | 19 | 53 | | |
| MulSatG63943 | $(CCT)_{10}$ | F: TTCAGATACCGTCCCCGAG | 61.0 | 19 | 58 | 230 | Pr032825895 |
| | | R: GGTGGTTAAGGCAAGTGGAAG | 60.9 | 21 | 52 | | |
| MulSatG9113 | $(ATA)_{10}$ | F: CAACTTTGGCTTCATCCACC | 60.5 | 20 | 50 | 198 | Pr032825901 |
| | | R: CCTACGCCCTTGTTATTGAGAG | 60.1 | 22 | 50 | | |
| MulSatG98364 | $(TAT)_{10}$ | F: AATTACGTCCGGGTTCAGG | 59.8 | 19 | 53 | 220 | Pr032825905 |
| | | R: CGAATCATCGCACTTTTGC | 60.4 | 19 | 47 | | |
| MulSatG13829 | $(TCT)_9$ | F: TCTCAGGCAGCTCATCTTCA | 59.8 | 20 | 50 | 266 | Pr032825878 |
| | | R: CGGAGAACACAAACTGGACA | 59.7 | 20 | 50 | | |
| MulSatG162762 | $(TCT)_9$ | F: ATTACATTACACCGCCCGAC | 59.7 | 20 | 50 | 202 | Pr032825884 |
| | | R: CCTTCCCAAAAGAGCAGGTT | 60.6 | 20 | 50 | | |
| MulSatG151860 | $(TCTT)_6$ | F: ACTGCGTTCACAGCATCCAAAG | 64.8 | 22 | 50 | 223 | Pr032825882 |
| | | R: GGTGGAGAATCAAAACCCAAGG | 63.9 | 22 | 50 | | |
| MulSatG44793 | $(TATT)_6$ | F: GTAGGTTGGGGAGTTGGAATCT | 60.6 | 22 | 50 | 228 | Pr032825890 |
| | | R: ACCTGGCATCCATAAACCC | 59.6 | 19 | 53 | | |

**Table 2.** Optimized PCR conditions and marker polymorphism observed in 216 germplasm.

| Marker | P | $T_A$ | C | V | Size range | $N_A$ | $N_R$ | $N_C$ | Rp |
|---|---|---|---|---|---|---|---|---|---|
| MulSatG100717 | 1 | 47 | 30 | 1 | 233–236 | 2 | 1 | 0 | 0.11 |
| MulSatG107143 | 2 | 51 | 30 | 3 | 203–245 | 6 | 3 | 0 | 1.65 |
| MulSatG107923 | 0.5 | 48 | 25 | 5 | 138–190 | 11 | 4 | 0 | 3.34 |
| MulSatG110731 | 0.25 | 48 | 30 | 5 | 194–244 | 10 | 6 | 0 | 2.52 |
| MulSatG118804 | 0.5 | 46 | 25 | 5 | 250–300 | 7 | 4 | 0 | 1.91 |
| MulSatG135883 | 1 | 48 | 25 | 5 | 196–248 | 14 | 6 | 0 | 3.05 |
| MulSatG140006 | 0.5 | 49 | 30 | 10 | 277–331 | 13 | 4 | 0 | 3.19 |
| MulSatG143363 | 0.75 | 48.5 | 30 | 3 | 178–253 | 8 | 2 | 0 | 2.26 |
| MulSatG149732 | 0.25 | 49 | 25 | 5 | 209–241 | 7 | 1 | 0 | 3.08 |
| MulSatG155763 | 0.25 | 50.5 | 30 | 10 | 248–292 | 12 | 6 | 0 | 2.35 |
| MulSatG178203 | 0.25 | 47 | 30 | 5 | 142–181 | 7 | 3 | 0 | 1.21 |
| MulSatG192454 | 0.5 | 50 | 30 | 3 | 146–229 | 11 | 5 | 0 | 3.00 |
| MulSatG44793 | 0.25 | 47 | 30 | 3 | 225–297 | 8 | 2 | 0 | 2.05 |
| MulSatG5166 | 1 | 50 | 30 | 3 | 147–153 | 3 | 2 | 1 | 0.12 |
| MulSatG52471 | 0.5 | 48.5 | 30 | 5 | 146–192 | 13 | 4 | 0 | 2.22 |
| MulSatG63943 | 0.5 | 48.5 | 30 | 5 | 205–223 | 5 | 3 | 1 | 0.43 |
| MulSatG79725 | 0.25 | 50 | 30 | 5 | 158–184 | 9 | 3 | 0 | 3.60 |
| MulSatG83251 | 0.25 | 52.5 | 35 | 3 | 156–196 | 14 | 7 | 0 | 2.59 |
| MulSatG86682 | 0.5 | 50.5 | 25 | 3 | 244–286 | 9 | 7 | 0 | 0.55 |
| MulSatG9113 | 0.25 | 48.5 | 30 | 3 | 204–216 | 5 | 2 | 0 | 1.19 |
| MulSatG92497 | 0.25 | 50 | 30 | 10 | 238–331 | 18 | 9 | 0 | 3.67 |
| MulSatG94574 | 0.5 | 50 | 30 | 3 | 180–189 | 4 | 1 | 0 | 1.13 |
| MulSatG95145 | 0.5 | 48 | 30 | 5 | 218–258 | 12 | 5 | 0 | 3.32 |
| MulSatG98364 | 0.3 | 45 | 30 | 10 | 205–274 | 8 | 1 | 0 | 2.64 |

P, optimized concentration of primers (pmol); $T_A$, optimized annealing temperature (°C); C, optimized number of PCR cycles; V, volume of PCR products loadded onto the gels ($\mu$L); $N_A$, number of alleles; $N_R$, number of rare alleles; $N_C$, number of common alleles; Rp, resolving power.

A set of 10 diverse mulberry accessions was used for screening the primers and optimizing PCR conditions. The PCR amplifications were carried out in 10 $\mu$L reaction volume containing 10 ng template DNA, 1× DreamTaq Green Buffer (Thermo Fisher Scientific, Vilnues, Lithuania), 0.1 mM of each dNTPs, 1 pmol of each forward and reverse primers and 0.25 U GeNei *Taq* DNA polymerase (Merck, Mumbai, India) on GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA) programmed to the following cycling profile: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s denaturation, primer specific annealing temperature (average $T_M$ of the forward and reverse primers –5) for 30 s and 72°C for 1 min extension, followed by the final extension step at 72°C for 8 min. Some primers failed to produce any amplification products and nonspecific amplification was observed in others. The problem was resolved by optimizing the primer concentration, number of PCR cycles and annealing temperature. Further, 216 mulberry germplasm were genotyped as above with optimized PCR conditions (table 2). The PCR products were resolved on 8% nondenaturing polyacrylamide gels according to the method of Sambrook and Russell (2001) using the Mega-Gel high-throughput electrophoresis system (CBS Scientific, San Diego, USA). pBR322 DNA-*Msp*I digest ladder (60 ng; New England BioLabs, Ipswich, USA) was used as a size standard. The gels were subjected to electrophoresis at 200 V for 4 h. SSR alleles were visualized by silver staining according to the protocol of Sanguinetti *et al.* (1994). The gels were then documented using CanoScan FB1210U flatbed scanner (Canon, Beijing, China).

Allele sizes of the microsatellite markers were estimated by comparing the bands to the pBR322 DNA-*Msp*I digest ladder. Allele informativeness ($I_b$) and resolving power (Rp) of each primer pair was calculated as per Prevost and Wilkinson (1999) using the formula $I_b = 1 - (2 \times |0.5 - p|)$, where p is the proportion of the germplasm containing the allele, and $Rp = \Sigma I_b$. The number of germplasm identified by a primer pair (x) was given by $x = (Rp - 1.78)/0.15$. Dice dissimilarity matrix of 216 mulberry germplasm was computed with the software DARwin ver. 6.0.14 (Perrier X. and Jacquemoud-Collet J. P. 2006 DARwin software http://darwin.cirad.fr/darwin). Discriminant analysis of principal components (DAPC; Jombart *et al.* 2010) was performed for assessment of population structure in the germaplasm using adegenet ver. 2.0.1 (Jombart 2008). Cluster analysis was performed using the data generated by 15 MulSatG markers with $Rp > 2$ by neighbour-joining method to check the degree of resolution that could be achieved.

## Results and discussion

Sericulture is a cottage industry that employs 8.03 million people across India and generates USD 312.12 million in foreign exchange annually for the exchequer (CSB 2017 Annual report 2016–17, Central Silk Board, Bengaluru). Mulberry is a perennial tree which is plagued by the constraints of tree crop breeding, namely out-crossing nature, heterozygosity, long juvenile period etc. Improvement of mulberry for yield, leaf quality, tolerance to abiotic stresses and resistance to biotic stress will help sustain the sericulture industry in the long run. Modern breeding approaches like marker-assisted selection help to target specific traits and speed up the process of cultivar improvement. Due to the lack of crop-specific codominant markers most of the genetic analysis in mulberry has relied on dominant markers (Vijayan *et al.* 2014). However, only recently, a reasonable number of SSRs were made available to researchers, but their application is still limited mainly to diversity analysis.

Of the 37 primer pairs designed in the present study, 13 failed to amplify any PCR products. PCR conditions were optimized for the remaining 24 primers and they were employed to genotype the 216 diverse mulberry germplasm. A total of 216 alleles in the size range 138–331 bp were observed across the germplasm, with an average of nine alleles/locus. MulSatG92497 generated the maximum of 18 alleles were as only two alleles were observed at the locus MulSatG100717. A total of 91 rare alleles (present in < 5% of the germplasm) and two common alleles (present in > 98% of the germplasm) were recorded (table 2). The maximum dissimilarity value of 0.893 was observed between Rajouri and Jalalgarah-3. The minimum dissimilarity of 0.015 was recorded between two accessions of *M. multicaulis*, namely ME-0006 and ME-0168. The average dissimilarity was found to be 0.547. The SSR genotypic data placed onto the panel of diverse germplasm can be utilized in the future as a molecular identification key, in genome mapping studies etc.

DAPC grouped the germplasm into three subpopulations (figure 1). The first subpopulation consisted 146 accessions mostly of *M. alba*, exotic species and some *M. indica* accessions. The second subpopulation was represented by 60 accessions of *M. indica*, *M. multicaulis* and a few accessions of *M. alba*. The third subpopulation was made up of 10 accessions belonging to the wild mulberry species *M. laevigata* and its hybrids. This is in concurrence with the known genetic relationships (Pinto *et al.* 2012). The germplasm material used in this study represents the diversity available in the gene bank (Krishnan 2014) and with correction for population structure, this set of germaplasm can be employed in association mapping for tagging genes/QTLs.

The resolving power of the SSR markers ranged from 0.11 (MulSatG100717) to 3.67 (MulSatG92497), with an average of 2.13 and totalling to 51.18. About eight

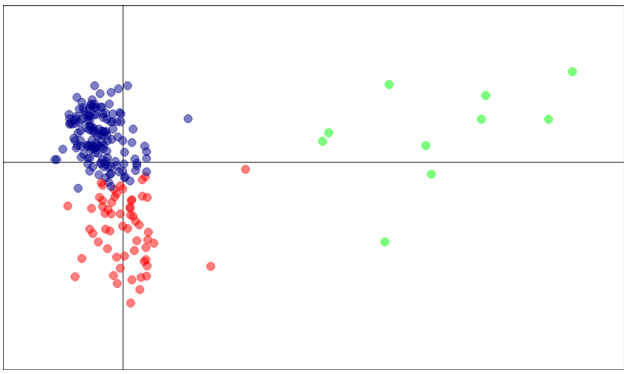

**Figure 1.** Clustering of diverse mulberry germplasm by discriminant analysis of principal components

markers had Rp > 3 and seven had Rp > 2. The cumulative Rp of these 15 markers was 42.88, which is theoretically sufficient to discern 274 germplasm accessions. All the 216 germplasm could be distinguished using the 15 SSR markers with Rp > 2. Further, these markers could also resolve 38 important mulberry cultivars (figure 2) that have a narrow genetic base (Krishnan *et al.* 2014b).

Protection of plant varieties and breeders' rights has taken the forefront these days. Distinctness, uniformity and stability (DUS) test guidelines for mulberry were developed recently at Central Sericultural Research and Training Institute, Mysuru under the aegis of Protection of Plant Varieties and Farmers' Right Authority, Govt. of India, New Delhi (http://plantauthority.gov.in/pdf/Mulberry.pdf). However, phenotypic plasticity in mulberry may hamper reliable distinction between cultivars as these characters are affected by factors like cultivation practices, environment and age of the plant (Gray 1990; Gray and Call 1994). DNA markers are neutral to such confounding factors and can be used for precise identification of germplasm, and to discern closely related cultivars having less phenotypic variability (Akagi *et al.* 1997). The hyper variability of SSRs (Powell *et al.* 1996) and their robustness in terms of reproducibility (Jones *et al.* 1997) makes them an excellent choice as molecular markers for cultivar identification. Genetic variation within a cultivar is unlikely since mulberry is clonally propagated. As per the existing guidelines, it takes about three years for conducting DUS test for a mulberry cultivar, and the test has to be conducted at two locations to account for genotype × environment interactions. This requires a huge investment in terms of time, manpower, land and farm inputs. Further, the limited number of phenotypic traits available for characterization will not be sufficient for establishing distinctness as the number of cultivars for testing increases. These problems can be largely circumvented by the use of SSR-based DNA fingerprinting as a diagnostic tool. Therefore, DNA fingerprinting using the validated SSR markers with high resolving power can

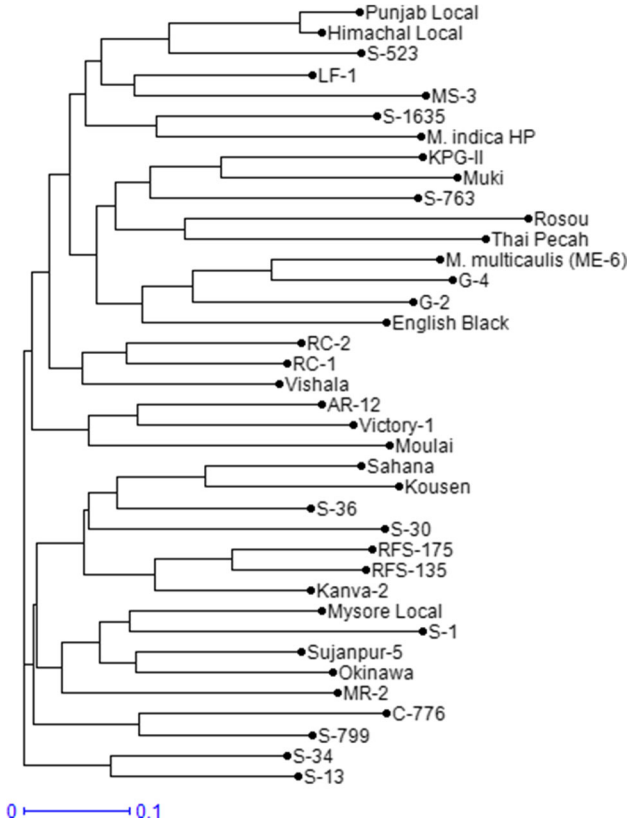


**Figure 2.** Neighbour-joining tree of the important mulberry cultivars based on 15 MulSatG (Rp > 2) marker data.

supplement the DUS test substantially for establishing distinctness of a new cultivar or for reliable, cost-effective and quick germplasm identification.

#### Acknowledgements

This study was funded by the Department of Biotechnology, Ministry of Science and Technology, Govt. of India, New Delhi grant no. BT/PR5811/PDB/19/238/2012 sanctioned to VGN. MVP was supported with Senior Research Fellowship by the Council of Scientific and Industrial Research, New Delhi. The authors gratefully acknowledge the help received from Mr M. S. Rukmangada, CSRTI, Mysuru during genomic DNA extraction and QC. The authors also thank Prof. H. S. Prakash, Department of Studies in Biotechnology, University of Mysore, Mysuru for extending the NanoDrop 2000C facility.

## References

Aggarwal R. K., Udaykumar D., Hendre P. S., Sarkar A. and Singh, L. I. 2004 Isolation and characterization of six novel microsatellite markers for mulberry (*Morus indica*). *Mol. Ecol. Notes* **4,** 477–479.

Akagi H., Yokozeki Y., Inagaki A. and Fujimura T. 1997 Highly polymorphic microsatellites of rice consist of AT repeats, and a classification of closely related cultivars with these microsatellite loci. *Theor. Appl. Genet.* **94**, 61–67.

Gao L. 2013 Development of EST-SSR primers in mulberry. *J. Southern Agri*. **44**, 1254–1257.

Gray E. 1990 Evidence of phenotypic plasticity in mulberry (*Morus* L.). *Castanea* **55**, 272–281.

Gray E. and Call N. M. 1994 Effects of induced plant injury on leaf lobation in red mulberry (*Morus rubra* L.). *Castanea* **59**, 167–175.

He N., Zhang C., Qi X., Zhao S., Tao Y., Yang G. *et al.* 2013 Draft genome sequence of the mulberry tree *Morus notabilis*. *Nat. Commun.* **4**, 2445.

Jombart T. 2008 adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* **24**, 1403–1405.

Jombart T., Devillard S. and Balloux F. 2010 Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genet.* **11**, 94.

Jones C. J., Edwards K. J., Castaglione S., Winfield M. O., Sala F., van de Wiel C. *et al*. 1997 Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Mol. Breed.* **3**, 381–390.

Kibbe W. A. 2007 OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Res.* **35**, W43–W46.

Krishnan R. R. 2014 Development of panel of diverse germplasm and core subset of mulberry (*Morus* spp.) by microsatellite marker aided analysis. Ph.D. Thesis, University of Mysore, Mysuru.

Krishnan R. R., Sumathy R., Bindroo B. B. and Naik V. G. 2014a MulSatDB: a first online database for mulberry microsatellites. *Trees* **28**, 1793–1799.

Krishnan R. R., Naik V. G., Ramesh S. R. and Qadri S. M. H. 2014b Microsatellite marker analysis reveals the events of the introduction and spread of cultivated mulberry in the Indian subcontinent. *Plant Genet. Resour.* **12**, 129–139.

Martins W. S., Lucas D. C. S., Neves K. D. S. and Bertioli D. J. 2009 WebSat – a web software for microsatellite marker development. *Bioinformation* **3**, 282–283.

Mathithumilan B., Kadam N. N., Biradar J., Reddy S. H., Ankaiah M., Narayanan M. J. *et al*. 2013 Development and characterization of microsatellite markers for *Morus* spp. and assessment of their transferability to other closely related species. *BMC Plant Biol.* **13**, 194.

Pinto M. V., Naik V. G. and Qadri S. M. H. 2012 Genetic variability studies in mulberry using microsatellite markers. *J. Sericult. Technol.* **3**, 38–43.

Powell W., Morgante M., Andre C., Hanafey M., Vogel J., Tingey, S. *et al*. 1996 The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Mol. Breed.* **2**, 225–238.

Prevost A. and Wilkinson M. J. 1999 A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theor. Appl. Genet.* **98**, 107–112.

Sambrook J. and Russell D. W. 2001 *Molecular cloning: a laboratory manual*, 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanguinetti C. J., Dias N. E. and Simpson A. J. 1994 Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *Biotechniques* **17**, 914–921.

Tani N., Kawahara T. and Yoshimaru H. 2005 Development and diversity of microsatellite markers for endangered species, *Morus boninensis* Koidz., to establish conservation program. *Mol. Ecol. Notes* **5**, 398–400.

Thumilan B. M., Sajeevan R. S., Biradar J., Madhuri T., Nataraja K. N. and Sreeman S. M. 2016 Development and characterization of genic SSR markers from Indian mulberry transcriptome and their transferability to related species of Moraceae. *PLoS One* **11**, e0162909.

Vijayan K., Raju P. J., Tikader A. and Saratchnadra B. 2014 Biotechnology of mulberry (*Morus* L.) – a review. *Emir. J. Food Agric.* **26**, 472–496.

Zhao W., Miao X., Jia S., Pan Y. and Huang Y. 2005 Isolation and characterization of microsatellite loci from the mulberry, *Morus* L. *Plant Sci.* **168**, 519–525.

Corresponding editor: L. S. SHASHIDHARA