ONLINE RESOURCES

A new set of validated SSR primers for application in mulberry cultivar identification with specific implication in 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 – 570008, India*

[2]*Regional Sericultural Research Station, Nanjangud Road, Chamarajanagar – 571313, India*

*For correspondence. E-mail: vgirishnaik@gmail.com

Short running title. Validated SSR primers for *Morus* spp.

Keywords. SSR, mulberry, cultivar identification, genetic analysis, genotyping, DUS testing.

Introduction

The monophagous domesticated silkworm *Bombyx mori* derives all its nutritional requirements from the mulberry (*Morus* spp.) leaves. Therefore, mulberry improvement has a key role in sustainably improving the quality and yield of silk. Lack of co-dominant markers for use in genetic mapping has constrained the efforts in marker assisted breeding for targeted trait improvement. Currently, a total of 417 mulberry specific SSR markers (160 genomic and 257 genic) are available for genome analysis (Aggarwal *et al*. 2004; Tani *et al*. 2005; Zhao *et al*. 2005; 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 the SSRs available in the draft 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.

Materials and methods

We chose 37 genomic sequences that had high number of 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) with default settings. OligoCalc Ver. 3.27 (Kibbe 2007) was used to calculate the T_M, length, GC content and to rule-out self-complementarity of 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^{TM} Plant Genomic DNA Miniprep Purification Kit (HiMedia Laboratories, India) following the manufacturer's instructions. A small aliquot of the DNA sample was run on 0.8% agarose gel in 1X TAE stained with Ethidium Bromide. The DNA was quantified using NanoDrop^{TM} 2000C (ThermoFisher Scientific, 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 10 ng/µl with $T_{10}E_1$ buffer and used in PCR.

A set of ten diverse mulberry accessions was used for screening the primers and standardizing PCR conditions. The PCR amplifications were carried out in 10 µl reaction volume, containing 10 ng template DNA, 1X DreamTaq^{TM} Green Buffer (ThermoFisher Scientific, Lithuania), 0.1 mM of each dNTPs, 1 pmol of each forward and reverse primers and 0.25 U *Taq* DNA polymerase (New England BioLabs, USA) on GeneAmp PCR System 9700 (Applied Biosystems, 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 of 72°C for 8 min. Some primers failed to produce any amplification products and non-specific amplification was observed in others. The problem was resolved by optimizing the primer concentration, number of PCR cycles and annealing temperature. Further, 216 mulberry accessions were genotyped as above with optimized PCR conditions (Table 2). The PCR products were

resolved on 8% non-denaturing polyacrylamide gels according to the method of Sambrook and Russel (2001) using the Mega-Gel System (CBS Scientific, USA). pBR322 DNA-*Msp*I digest ladder (New England BioLabs, USA) was used as a size standard (60 ng/lane). The gels were subjected to electrophoresis at 200 V for 4 hours. SSR alleles were visualised by silver staining according to the protocol of Sanguinetti *et al*. (1994). The gels were then documented using CanoScan FB1210U flatbed scanner (Canon, China).

The allele sizes of the microsatellite markers were estimated by comparing the bands generated 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) with 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 accessions identified by a primer pair (x) was given by $x = (Rp - 1.78)/0.15$. Dice dissimilarity matrix of 216 mulberry accessions was computed with the software DARwin Ver. 6.0.14 (Perrier and Jacquemoud-Collet 2006). Discriminant analysis of principal components (DAPC; Jombart *et al*. 2010) was performed for assessment of population structure in the germaplasm accessions using the adegenet package for R (Jombart 2008). Cluster analysis was performed using the data generated by 15 MulSatG primers with Rp >2 by Neighbour Joining method to check for 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 US$ 389.53 million in foreign exchange annually for the exchequer (CSB 2016). Mulberry is a perennial tree that is plagued by the constraints of tree crop breeding *viz*. 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 co-dominant 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 current 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 accessions. A total of 216 alleles

in the size range 138-331 bp were observed across the germplasm, with an average of 9 alleles/primer pair. The primer MulSatG92497 generated the maximum of 18 alleles were as only 2 alleles were observed in MulSatG100717. A total of 91 rare alleles (present in <5% of the germplasm) and 2 common alleles (present in >98% of the germplasm) were observed (Table 2). The maximum dissimilarity value of 0.893 was observed between the genotypes Rajouri and Jalalgarah-3. The minimum dissimilarity of 0.015 was recorded between two accessions of *M. multicaulis viz.* 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 for development of molecular IDs, in genome mapping studies *etc*.

DAPC grouped the germplasm accessions into 3 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 genome wide association studies 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 8 primers had Rp >3 and 7 had Rp >2. The cumulative Rp of these 15 primers was 42.88, which can theoretically differentiate between 274 germplasm accessions. All the 216 germplasm could be distinguished using the 15 SSR primers with Rp >2. Further, these primers could also resolve 38 popular 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. DUS test guidelines for mulberry was 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 reliably used to identify cultivars, including closely related individuals which may have less morpho-phenotypic variability (Wünsch and Hormaza

2002). The hyper variability of SSRs (Powell *et al*. 1996) and robustness in terms of reproducibility (Jones *et al*. 1997) makes them an excellent choice as molecular markers for cultivar identification. As the crop is clonally propagated, genetic variation within a cultivar is unlikely. Currently, it takes about 3 years for conducting DUS test for a mulberry variety, and has to be conducted at 2 locations to account for genotype × environment interactions. This requires a huge investment in terms of time, manpower, land and farm inputs. These problems can be alleviated largely by the use of SSR based DNA fingerprinting as a diagnostic tool. Therefore, we propose that the 15 SSR markers (with Rp >2) can be used for reliable identification of mulberry cultivars and even to distinguish between closely related varieties in tandem with DUS testing.

Acknowledgements

MVP acknowledges the financial support received from the Council of Scientific and Industrial Research, New Delhi in the form of Senior Research Fellowship. The study was funded with the Department of Biotechnology, New Delhi grant No. BT/PR5811/PDB/19/238/2012 sanctioned to VGN. 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, Chairman, Department of Studies in Biotechnology, University of Mysore, Mysuru for extending the NanoDrop™ 2000C facility.

Authors' contributions

VGN and MVP conceived the study. HSP and VGN designed the primers. MVP and HSP standardized 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.

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.

CSB. 2016 *Annual Report 2015-16*. Central Silk Board, Bengaluru.

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., 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.

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.

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.

Perrier, X. and Jacquemoud-Collet, J.P. 2006 DARwin software http://darwin.cirad.fr/darwin

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*. 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.

Wünsch, A. and Hormaza, J. I. 2002 Cultivar identification and genetic fingerprinting of temperate fruit tree species using DNA markers. *Euphytica*, **125**, 59-67.

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.

Unedited version Published Online 11 January 2018

Figure legends

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

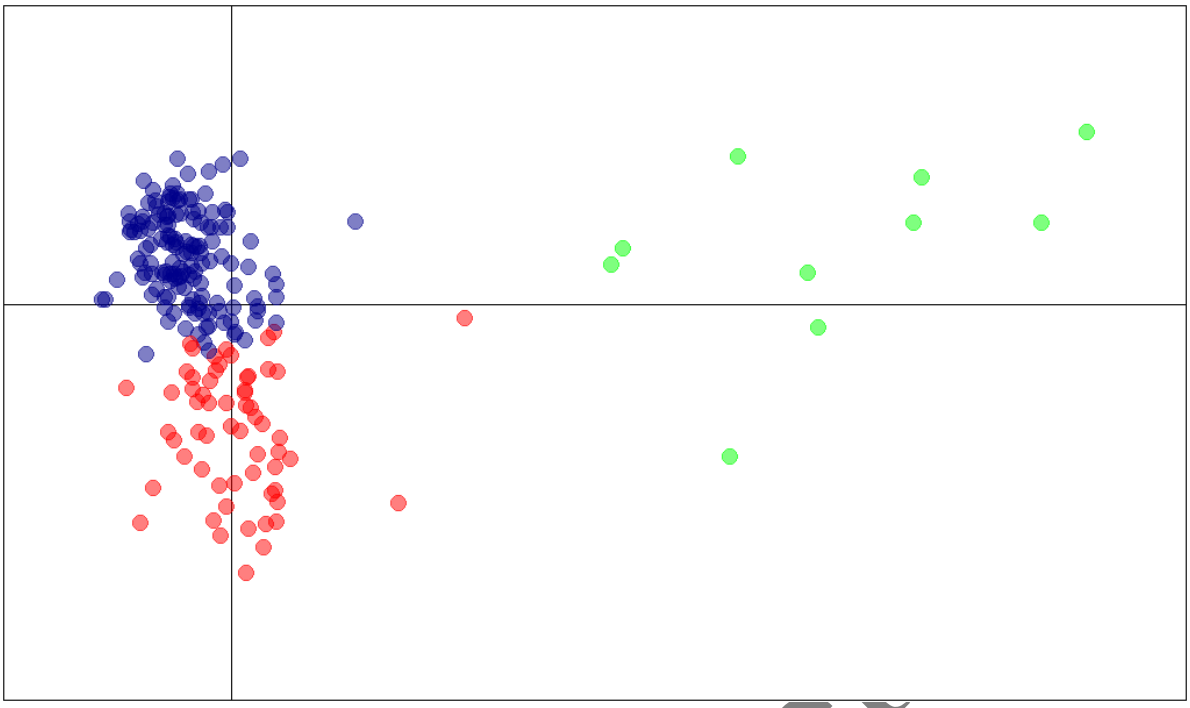

Figure 2. Neighbour Joining tree of popular mulberry cultivars distinguished by the 15 SSR markers with Rp >2

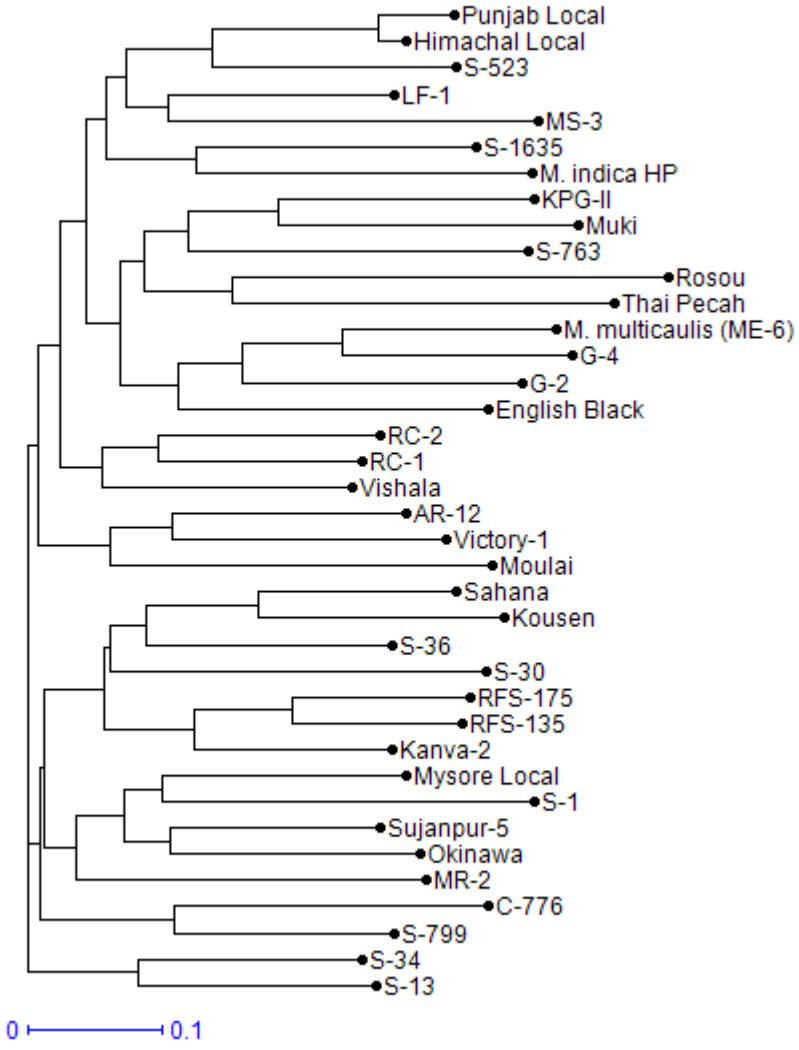
Punjab Local
Himachal Local
S-523
LF-1
MS-3
S-1635
M. indica HP
KPG-II
Muki
S-763
Rosou
Thai Pecah
M. multicaulis (ME-6)
G-4
G-2
English Black
RC-2
RC-1
Vishala
AR-12
Victory-1
Moulai
Sahana
Kousen
S-36
S-30
RFS-175
RFS-135
Kanva-2
Mysore Local
S-1
Sujanpur-5
Okinawa
MR-2
C-776
S-799
S-34
S-13
0
0.1

Table 1. MulSatG – a new series of mulberry specific SSR markers.

Marker name	Repeat motif	Primer sequence (5'→3')	T_M	Length	GC%	Product size	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		

Table 1 (*contd*)

Marker name	Repeat motif	Primer sequence (5'→3')	T_M	Length	GC%	Product size	NCBI ProbeDB Acc. No.
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		
MulSatG172234	$(CT)_{22}$	F: GACAGTGCTCAACAACCACG	60.4	20	55	219	Pr032825886
		R: GGGGAGGGATTAGAGAGAGAGA	60.2	22	55		
MulSatG75021	$(CA)_{22}$	F: CGATATTGGCTAGGAACGG	58.2	19	53	177	Pr032825896
		R: CCCACAGATAAAACCCCATC	59.1	20	50		

Table 1 (*contd*)

Marker name	Repeat motif	Primer sequence (5'→3')	T_M	Length	GC%	Product size	NCBI ProbeDB Acc. No.
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		

Table 1 (*contd*)

Marker name	Repeat motif	Primer sequence (5'→3')	T_M	Length	GC%	Product size	NCBI ProbeDB Acc. No.
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	0.25	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	4	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 loaded onto the gels (µl); N_A – number of alleles; N_R – number of rare alleles; N_C – number of common alleles; Rp – resolving power.