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Suraksha Chanotra
Ph.D., Scholar, Div. of Sericulture, SKUAST-J, Chatha, Jammu, Jammu and Kashmir, India

RK Bali
Professor, Div. of Sericulture, SKUAST-J, Chatha, Jammu, Jammu and Kashmir, India

RK Salgotra
Professor, School of Biotechnology, SKUAST-J, Jammu, Jammu and Kashmir, India

K Bali
Associate Professor, Div. of Sericulture, SKUAST-J, Chatha, Jammu, Jammu and Kashmir, India

GK Rai
Associate Professor, School of Biotechnology, SKUAST-J, Chatha, Jammu, Jammu and Kashmir, India

Correspondence
Suraksha Chanotra
Ph.D., Scholar, Div. of Sericulture, SKUAST-J, Chatha, Jammu, Jammu and Kashmir, India

Evaluation of genetic diversity in selected *Morus* genotypes using molecular markers

Suraksha Chanotra, RK Bali, RK Salgotra, K Bali and GK Rai

Abstract
An attempt was carried out in the present investigation to estimate the extent of genetic diversity and relationship among forty four mulberry genotypes using SSR markers. A set of 26 SSR primers were used to assess the genetic diversity which generated a total of 182 bands with an average of 6.96 alleles per locus. Primer M6 produced maximum of 15 alleles per locus and minimum of 3 alleles per locus in primer Mul3SSR91. Cluster tree grouped 44 genotypes into four main clusters, A, B, C and D with sub-clusters and sub-sub clusters. Highest PIC value was recorded for SSR marker M6 (0.901) and lowest for primer Mul3SSR91 (0.486) with an average of 0.724. Genotype, NS-1 and NS-2 were identified as duplicates while other genotypes showed least similarity and recorded availability of diversity among germplasm.

Keywords: Mulberry, genetic diversity, simple sequence, cluster tree, duplicates

Introduction
The importance of mulberry from industrial point of view can be gauged from the fact that its foliage forms the only natural food of silkworm (*Bombyx mori* L). The production and productivity of silk hence demands an improvement of the host plant manifested in terms of quantitative and qualitative leaf production (Arora *et al.,* 2014) [1]. In India apart from sericulture purposes, diverse genetic resources of mulberry have also been exploited for multipurpose usage (Tikader and Roy 2006) [7].
Over the years, molecular markers have been extensively used to characterize the mulberry cultivars because they are plentiful, co-dominant in nature and are locus specific in nature. Additionally they are not affected by changing environment and growth stage of the genotype.
As it is prudent to subject the mulberry accessions to genetic characterization using molecular markers to understand the extent of genetic diversity and to eliminate duplicates and nearly similar accessions. In the present study, we employed SSR markers to characterize the mulberry genotypes so as to have a better understanding of genetic relationship of different genotypes.

Material and Methods
Leaf samples of the 44 selected varieties were collected from mulberry germplasm bank maintained at Divison of Sericulture, SKUAST-J, Udheywala campus (Table 1) and molecular analysis was conducted in the laboratory of School of Biotechnology, SKUAST-J, Chatha.

Genomic DNA isolation and quantification
Total genomic DNA was isolated using the modified CTAB (Cetyl trimethyl ammonium bromide) protocol by Doyle and Doyle (1990) [4]. The amount and quality of DNA was confirmed using Nanodrop. Finally the DNA was diluted to 25ng/µl concentration for PCR amplification.

Diversity analysis based on SSR markers
40 SSR markers were used in present investigation (Table 2) out of which 26 SSR primers produced polymorphic results and revealed diversity at molecular level.

Table 1: Details of Mulberry genotypes with their origin and genetic nature

S. No	Name of Genotype	Donor Name	Origin
1.	Asayuki	CSRTI, Mysore	Cross Pollinated Hybrid
2.	Enshutukasuka	CSR & TI, Mysore	Collection
3.	Fukushima	CSR & TI, Berhampore	Collection
4.	Goshyerami	CSR & TI, Mysore	Selection
5.	Ichinose	RSRS, Kodathi	Cross Pollinated Selection
6.	Kairyoroso	CSRTI, Mysore	Cross Pollinated Hybrid
7.	Kamabori	CSRTI, Mysore	Cross Pollinated Hybrid
8.	Kokuso-20	CSR & TI, Mysore	Mutation
9.	Kokuso-27	CSR & TI, Mysore	Cross Pollinated Hybrid
10.	Limencina	CSR & TI, Mysore	Collection
11.	Miuraso	CSR & TI, Mysore	Collection
12.	Rokokyoso	RSRS, Kodathi	Clonal Selection
13.	Shimanouchi	CSR & TI, Mysore	Cross Pollinated Hybrid
14.	BC-259	CSR & TI, Berhampore	Back Cross Selection
15.	Bhrem C-776	CSR & TI, Pampore	Cross Pollinated Selection
16.	Behrampur	CSR & TI, Berhampore	Clonal Selection
17.	C-763	CSRTI, Mysore	Cross Pollinated Hybrid
18.	Chakmajra	DOS, J & k Govt.	Natural Selection
19.	China white	CSR & TI, Mysore	Collection
20.	Dhar local	DOS, J & k Govt.	Open Pollinated Hybrid
21.	Kanva-2	CSR & TI, Mysore	Cross Pollinated Hybrid
22.	KNG	CSR & TI, Mysore	Clonal Selection
23.	LF-1	CSR & TI, Mysore	Clonal Selection
24.	LF-2	CSR & TI, Mysore	Clonal Selection
25.	NS-1	Div. of Sericulture, SKUAST-J.	Open Pollinated
26.	NS-2	Div. of Sericulture, SKUAST-J.	Open Pollinated
27.	NS-3	Div. of Sericulture, SKUAST-J.	Open Pollinated
28.	S-1	CSR & TI, Mysore	Clonal Selection
29.	S-30	CSR & TI, Mysore	Mutation
30.	S-36	CSR & TI, Mysore	Mutation
31.	S-41	CSR & TI, Mysore	Mutation
32.	S-54	CSR & TI, Mysore	Mutation
33.	S-146	RSRS, Kodathi	Open Pollinated Selection
34.	S-799	CSR & TI, Mysore	Open Pollinated Hybrid
35.	S-1531	CSR & TI, Mysore	Open Pollinated Selection
36.	S-1608	CSR & TI, Berhampore	Open Pollinated Hybrid
37.	S-1635	CSR & TI, Berhampore	OPH Selection
38.	S-1708	CSR & TI, Berhampore	Open Pollinated Selection
39.	Sujanpur	DOS, J&K Govt.	Open Pollinated Collection
40.	Tr-1	CSR & TI, Berhampore	Colchiploid
41.	Tr-4	RSRS, Kodathi	Polyploid
42.	Tr-8	RSRS, Kodathi	Polyploid
43.	Tr-10	RSRS, Kodathi	Polyploid
44.	V-1	CSRTI, Mysore	Cross Pollinated Hybrid

Table 2: List of SSR primers used

S. No	Primer	Sequence	Amplicon size (bp)
1	Mul3SSR4 F Mul3SSR4 R	GGAGCAGTCAATCTCTTG CTGGGGTTCAAACTAAGCTC	314
2	Mul3SSR9 F Mul3SSR9 R	GACCAGCCATGAGCCTAC GGTTCACAACCACAATCTCC	365
3	Mul3SSR16 F Mul3SSR16 R	CTAGTAGCAGATCACCAC CGGTCTCTCCCTAATCC	207
4	Mul3SSR17 F Mul3SSR17 R	GTCTTGCACTAGGAGAGG CTCACAGGAGAACACCACC	345
5	Mul3SSR19 F Mul3SSR19 R	CCAAGTCCTCCTCCAG GTTTTGTGACTTGCCG	170
6	Mul3SSR20 F Mul3SSR20 R	CTAGCAGATCGTGGCATTG CTCCGCCCAAAATATCACAC	252
7	Mul3SSR50 F Mul3SSR50 R	CTAGCAGATCCACCAAACC GTTGTTGTACTCTCGCACG	161
8	Mul3SSR53 F Mul3SSR53 R	CAGCTATGACCATGATTACGCC GGACCCTTGATGGCATTG	124
9	Mul3SSR65 F Mul3SSR65 R	CTGGAGTACAAGAACCGCAAC GCCCTCCACCATTGAACTAAG	210
10	Mul3SSR70 F Mul3SSR70 R	GAAGAGGGGAGAGGGAGAGA CAACCAGGATCCAAATAGAAGC	170
11	Mul3SSR71 F Mul3SSR71 R	GGATACTACCTGTTTGGTTGCTG ATTCCCTCCTCAACGAC	360
12	Mul3SSR74 F Mul3SSR74 R	CCCATTGAGGGTTTTGTGAG ATGTGAGCTCGGGATTTGAC	400
13	Mul3SSR80 F Mul3SSR80 R	GAGCCGTTTGATTTCCGTC CAACGGTCGGTGAAAAAGC	140
14	Mul3SSR91 F Mul3SSR91 R	CATGAACCGTTGGATCACAG ATCCCAGATCCCAAATACCC	227
15	Mul3SSR93 F Mul3SSR93 R	CAGCCAATGCACTTTTAACG GTGGAGCTTCTGTTGAGC	340
16	Mul3SSR94 F Mul3SSR94 R	CCCTCATGTGTTCCATCTACC CAGAATCACAGCCGAGGAAG	195
17	Mul3SSR95 F Mul3SSR95 R	GATCATCGTGCCAATAAGCC TAAGAGCTGAGAGGGGAAGC	209
18	Mul3SSR97 F Mul3SSR97 R	TCCACCACTGAACCAAATC ATTAGGGTTGTGACGACGAC	292
19	Mul3SSR102F Mul3SSR102R	TTGGTTGCTGAGAAATGCAG TTGTCGATGGAAAACACGAC	225
20	Mul3SSR103F Mul3SSR103R	GGTCAGATCAGTTTCGTTGC GTAAGAGCTGAGAGGGGAAG	235

21	Mul3SSR105F Mul3SSR105R	GCAGAATCCCAAGTTAATGCC CCTCATAGAGTACAGGAACCG	240
22	Mul3SSR114F Mul3SSR114R	GCAACTCTGCCTTGTTTTC TGGTGCCTTAGACCAGAC	102
23	Mul3SSR116F Mul3SSR116R	CCAAGGAAGGTGAAATCC CATGAACCGTTGGATCACAG	277
24	Mul3SSR122F Mul3SSR122R	GGTGATGGGCTTTTGATG GTTGGATCTGAGGAGGGTC	219
25	Mul3SSR131F Mul3SSR131R	ACTGTGCTTCGTGGAGTTG GAGAGCTTCGAGAGGGAGG	300
26	Mul3SSR187F Mul3SSR187R	GGACATTTCACAACCCTG AACTGCAAGTTGGCACAG	324
27	Mul3SSR197F Mul3SSR197R	GGTGAAAGTTCGTGTGAGTCC CAGCAACTAGAGTGACTTTG	180
28	Mul3SSR203F Mul3SSR203R	GACCGTAGGAGAGAGTGC GGATACCCGCTAAACCCAC	440
29	Mul3SSR229F Mul3SSR229R	CCTTATAGCCGATTTTGCAGGC GAAATTCCGACTCCATGGTC	240
30	Mul3SSR230F Mul3SSR230R	CGGGTGAGCTGGTTTGTTTC CAGCCCCACAATCCCTACT	380
31	SS05	F: TCCAGCAAAGATGTGACAAAAGTT R: TTGCCTTCCCGATTATGCTG	350
32	SS02	F: GCTTCGATCAATCTAGCTTCCC R: GCAAACTACGCCACCCCG	355
33	SS04	F: CGAGGGAGGGATGAGGAGC R: CACATTCATCCACCCTCCTATA	190
34	SS17	F: TACAGGGCTCGGGCAAATG R: TGATCCGAAGCTTGGGGTCT	220
35	SS06	F: ACTCAAAATGAAGGAAAAGGAATTATAC R: TTTACTTAAATCCCAGCCACA	180
36	SS19	F: TTCTGTCGTGTCCTCCGTCAA R: TGAGAACATACACTAATAGGTGAAAAC	300
37	SS09	F: AGAACCCTTCCGCCCTATG R: CCTTGGCGTAGGCAAAGTTG	200
38	SS18	F: TCTTCGCCCGTTGTTCGC R: AGCAATTTTCTTCAACTCACCTTCT	180
39	M2	F: CGTGGGCTTAGGCTGAGTAGAGG R: CACCACCACTACTTCTCTTCTTCCAG	190
40	M6	F: TCCTTAGGTTTTTGGGGTCTGTTTACAT R: CCTCATTCTCCTTTCACTTATTGTTG	310

Components of PCR reaction

Table 3: Reagents with their concentration and quantity used for single PCR reaction

S. No	Reagents	Concentration	Quantity
1	Tempelate DNA	2 ng/ µl	1 µl
2	Sterile water		0.6 µl
3	PCR Buffer	10X	1.5 µl
4	Mgcl2	2mM/µl	1.2 µl
5	dNTPs	0.2 mM/µl	1.5 µl
6	Primer	5pmole	4.5 µl (F and R each)
7	Taq Polymerase	5U/v	0.2 µl
	Total		15 µl

PCR amplification

Table 4: Thermal profile used for DNA amplification

Steps	Cycles	Temperature	Duration
Denaturation	1	95 ºC	5 min
Denaturation	30	95 ºC	1 min
Anneling		45-65 ºC	45 sec
Extension		72 ºC	45 sec
Final extension	1	72 ºC	8 min

Diversity analysis

Bi-nomial data matrix of all the genotypes generated from 40 primers was subjected to analysis using NTSYS pc version 2.11a software and certain parameters like polymorphism percentage and polymorphic information content were evaluated. Cluster tree was generated through Power marker analysis.

Results

182 alleles were recovered for 26 SSR markers when screened with 44 mulberry genotypes used in the study. The data with respect to allelic variation and frequency is presented in Table 5 and plate 1. The gel plates with respect to marker Mul3SSR122 clearly indicated the genetic diversity in the genotypes with respect to target loci. However, remaining twenty five markers also produced high degree of polymorphism for all studied genotypes. The number of alleles per locus ranged from 3 (Mul3SSR91) to 15 (M6) with an average of 6.96 alleles per locus. The major allele frequency varied from 0.136 (M6) to 0.558 (M2) with an average of 0.351. Highest allele frequency was observed with marker M2 (0.558) at band size of 190-210bp followed by Mul3SSR197 (0.548) at band size of 180-190bp and lowest in M6 (0.136) at 125-160 bp. Heterozygosity values for each primer ranged from 0.022 (Mul3SSR114) to 0.870 (Mul3SSR105) with a total average of 0.200. Percentage of polymorphism for studied primers ranged from 14.20 per cent (Mul3SSR80) to 100 per cent (Mul3SSR70, Mul3SSR74 and Mul3SSR114) with an average of 39.84 percent.

Table 5: Major allelic frequency, number of observations, allele number, availability, gene diversity, heterozygosity, polymorphism percentage and PIC content

S. No	Primer	Band size (bp)	Major allele frequency	No. of obs.	Allele no.	Availability	Gene diversity	Heterozygosity	PP	PIC
1.	Mul3SSR9	365	0.289	38	12	0.863	0.850	0.137	50.00	0.836
2.	Mul3SSR19	170	0.173	23	08	0.522	0.850	0.057	33.30	0.832
3.	Mul3SSR65	210	0.428	28	08	0.636	0.752	0.125	60.00	0.726
4.	Mul3SSR70	170	0.277	18	08	0.409	0.827	0.034	100.00	0.805
5.	Mul3SSR74	400	0.266	15	06	0.340	0.773	0.036	100.00	0.737
6.	Mul3SSR80	140	0.314	35	08	0.795	0.813	0.159	14.20	0.790
7.	Mul3SSR91	227	0.454	11	03	0.25	0.578	0.045	33.30	0.486
8.	Mul3SSR93	340	0.423	26	06	0.590	0.742	0.108	20.00	0.710
9.	Mul3SSR94	195	0.500	16	04	0.363	0.648	0.066	33.30	0.592
10.	Mlrl3SSR97	292	0.256	39	08	0.886	0.820	0.850	14.20	0.796
11.	Mul3SSR102	225	0.400	10	04	0.227	0.700	0.026	33.30	0.645
12.	Mul3SSR103	235	0.318	22	07	0.500	0.801	0.580	40.00	0.775
13.	Mul3SSR105	240	0.268	41	10	0.931	0.855	0.870	25.00	0.840
14.	Mul3SSR114	102	0.285	14	08	0.318	0.836	0.022	100.00	0.818
15.	Mul3SSR122	219	0.342	38	07	0.863	0.788	0.186	16.60	0.759
16.	Mul3SSR131	300	0.500	32	04	0.727	0.580	0.770	33.30	0.493

17.	Mul3SSR197	180	0.548	31	05	0.704	0.626	0.237	25.00	0.580
18.	Mul3SSR203	440	0.428	14	05	0.318	0.704	0.037	66.60	0.657
19.	Mul3SSR229	240	0.333	18	05	0.409	0.753	0.062	25.00	0.711
20.	Mul3SSR230	380	0.291	24	08	0.545	0.815	0.079	60.00	0.792
21.	SS05	350	0.294	34	07	0.772	0.807	0.023	16.60	0.781
22.	SS02	355	0.447	38	07	0.863	0.720	0.023	40.00	0.684
23.	SS19	300	0.333	21	06	0.477	0.780	0.072	20.00	0.748
24.	SS18	180	0.275	40	06	0.909	0.78	0.228	20.00	0.744
25.	M2	190	0.558	34	06	0.772	0.634	0.264	20.00	0.600
26.	M6	310	0.136	44	15	1	0.909	0.107	36.30	0.901
Mean		-	-	44	6.96	0.615	0.759	0.200	39.84	0.724

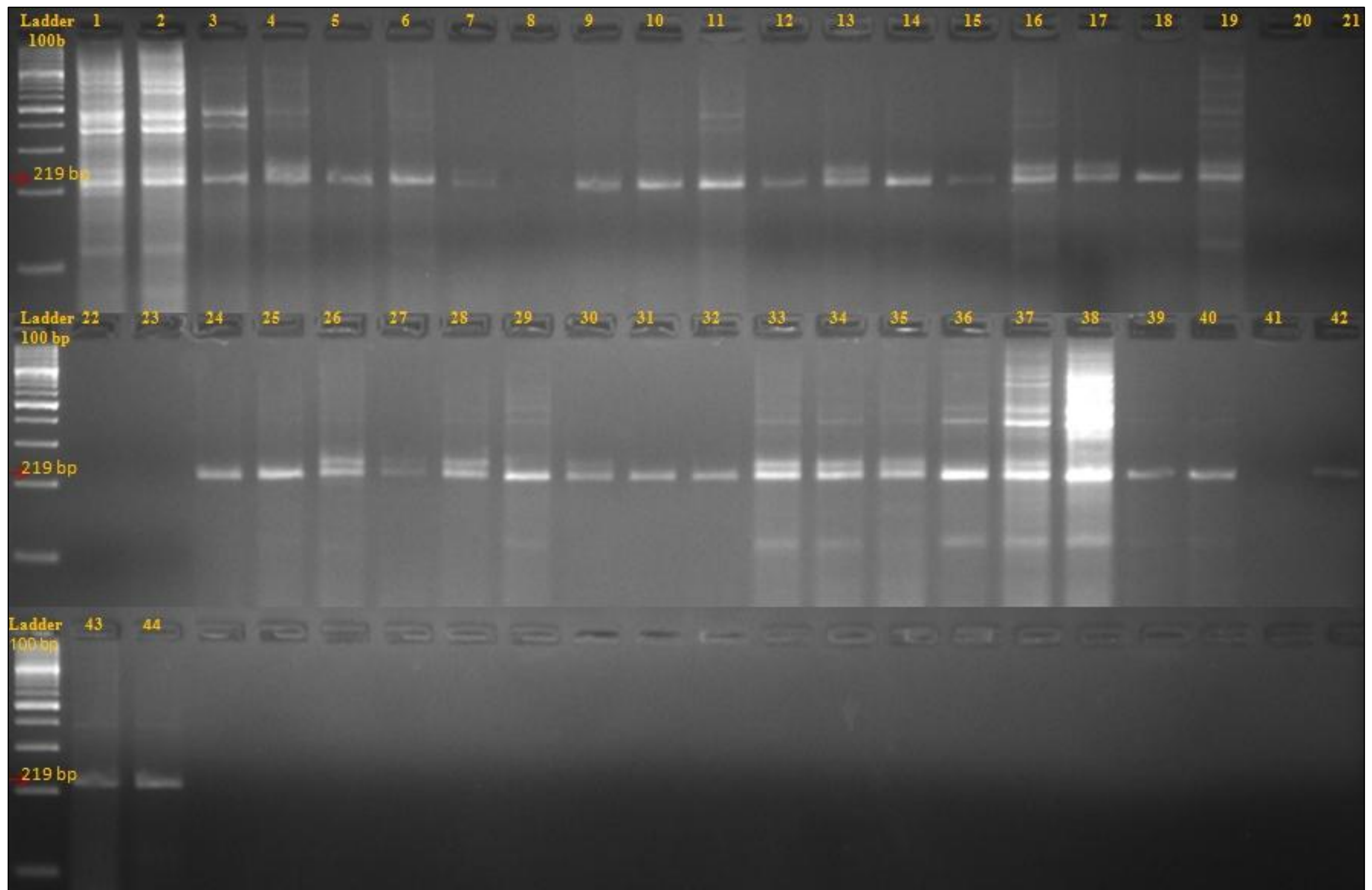

Plate 1: Showing PCR amplification of Mul3SSR122 at 219 bp

1-BC-259 2- Tr-10 3- Kanva-2 4-S-799 5-C-763 6-Asayuki 7-Fukushima 8-Kamabori 9-Chakmajra 10-Behrampur 11-S-1 12-Sujanpur 13-Goshyerami 14-Kokuso-20 15-Kairyoroso 16-Enshutukasuka 17-Rokokyoso 18-Kokuso-27 19-Shimanouchi 20-NS-2 21-NS-1 22-NS-3 23-Limencina 24-KNG 25-Chinese white 26-Ichinose 27-S-41 28-S-54 29-Tr-1, 30-Tr-4 31-Tr-8 32-LF-1 33-LF-2 34-S-35 34-S-30, 36-Miuraso 37-Dal local 38-Bhrem C-776 39-S-1608 40-S-1635 41-V-1 42-S-146 43-S-1708 and 44-S-1531.

Cluster tree analysis of SSR markers

The data was analysed using Power marker software and cluster tree was constructed which revealed a peculiar picture showing different clusters of genotypes based on genetic similarity. Cluster tree grouped fourty four genotypes under four main clusters A, B, C and D representing intensity of diversity and closeness among them (Table 6). Cluster-A comprised of two sub-clusters A_1 and A_2. Sub-cluster A_1 consists of BC-259 and Tr-10. Sub-cluster A_2 had two sub-sub clusters i.e. A_{2a} (Fukushima) and A_{2b} (S-1531 and S-1708). Cluster- B comprised of two sub-clusters i.e. B_1 and B_2. Sub-cluster-B_1 consists of Asayuki and C-763 and Sub-cluster-B_2 further divided into four sub-sub-clusters i.e. B_{2a}, B_{2b}, B_{2c} and B_{2d}. Sub-sub-cluster B_{2a} consists of three genotypes namely Kanva-2, S-799 and Sujanpur and B_{2b} consists of three genotypes *viz*. NS-3, Goshyerami and Kokuso-20. Sub-sub cluster B_{2c} comprised of Kokuso-27, Enshutukasuka and Rokokyoso and sub-sub-cluster B_{2d} consists of genotypes Behrampur, S-1, Kairyoso, Chakmajra and Kamabori. Cluster-C comprised of three sub-clusters i.e. C_1, C_2 and C_3. Sub-cluster-C_1 got divided into two sub-sub-clusters C_{1a} and C_{1b}. C_{1a} consists of S-1608 and S-1635 and sub-cluster C_{1b} consists of Miuraso, Bhrem C-776 and Dhar local. Sub-cluster C_2 comprised two sub-sub clusters C_{2a} (S-30) and C_{2b} (LF-2 and S-36) and sub-cluster C_3 had two sub-sub clusters i.e. C_{3a} (S-146) and C_{3b} (Tr-8, LF-1 and V-1). Cluster-D comprised of two sub-clusters D_1 and D_2. Sub-cluster D_1 had two sub-sub clusters D_{1a} and D_{1b} with genotypes Shimanouchi under D_{1a} and NS-1 and NS-2 under D_{1b}. Sub-clusters-D_2 comprised of four sub-sub-clusters i.e. D_{2a}, D_{2b}, D_{2c} and D_2d. Sub-cluster D_{2a} consists of Tr-1 and Tr-4, D_{2b} consists of S-41 and S-54, D_{2c} comprised of Chinese-white and Ichinose and D_{2d} KNG and Limencina as shown in Figure 1 and Table 6.

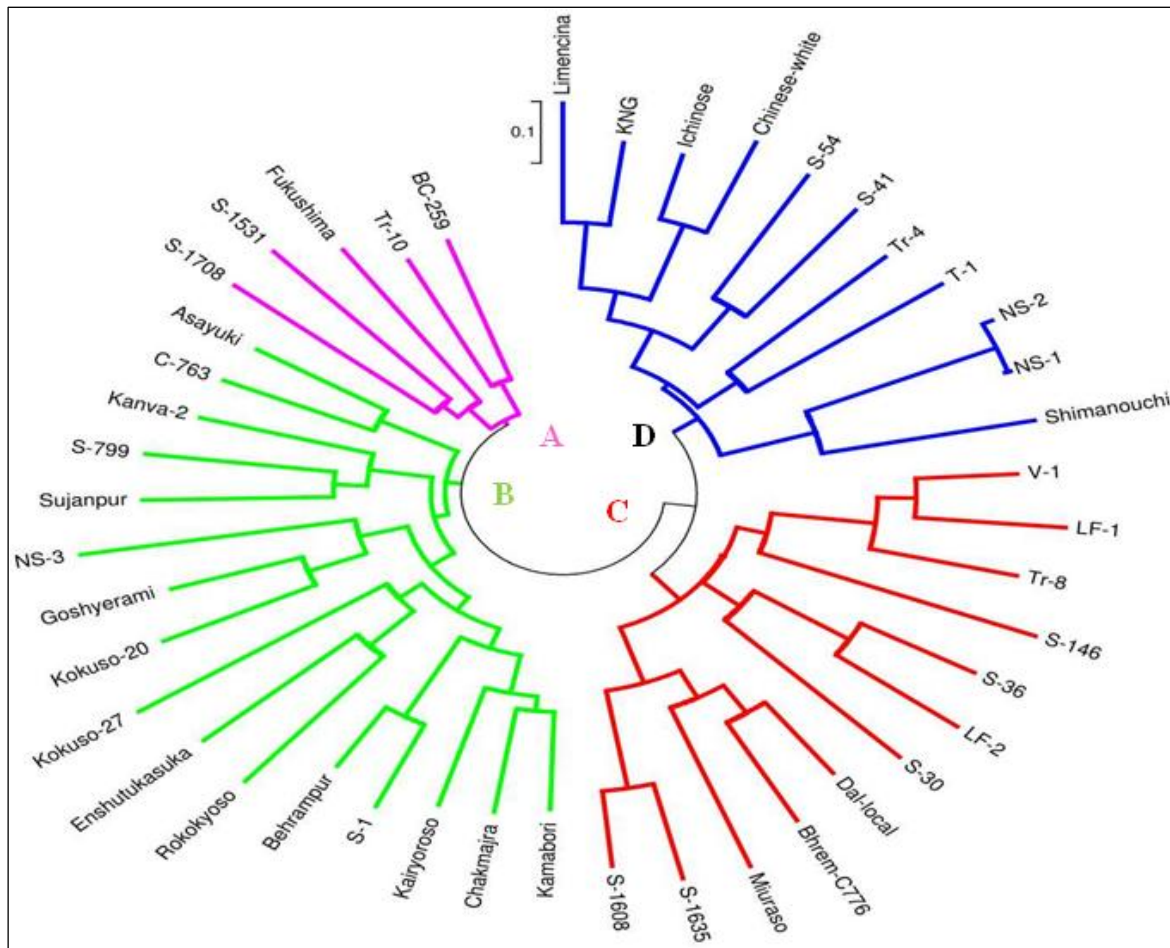

Fig 1: Cluster of 44 genotypes generated through Power marker

Table 6: Cluster tree analysis of SSR markers with Power marker software

S. No	No. of entries	Clusters	Sub-clusters	Sub- sub clusters	Genotypes
1	5	A	A_1 and A_2	A_{2a} and A_{2b}	BC-259, Tr-10, Fukushima, S-1531 and S-1708.
2	16	B	B_1 and B_2	B_{2a}, B_{2b}, B_{2c} and B_{2d}.	Asayuki, C-763, Kanva-2, S-799, Sujanpur, NS-3, Goshyerami, Kokuso-20, Kokuso-27, Enshutukasuka, Rokokyoso, Behrampur, S-1, Kairyoroso, Chakmajra, Kamabori.
3	12	C	C_1, C_2 and C_3	C_{1a}, C_{1b}, C_{2a} C_{2b}, C_{3a} and C_{3b}	S-1608, S-1635, Miuraso, Bhrem-C776, Dhar-local, S-30, LF-2, S-36, S-146, Tr-8, LF-1 and V-1.
4	11	D	D_1 and D_2	D_{1a}, D_{1b}, D_{2a}, D_{2b}, D_{2c} and D_{2d}.	Shimanouchi, NS-1, NS-2, Tr-1, Tr-4, S-41, S-54, Chinese-white, Ichinose, KNG and Limencina.
Total	44				

Discussion

In the present investigation, a total of 182 alleles were observed using 26 SSR markers when screened with 44 mulberry genotypes. Number of alleles detected for SSR markers varied from 3 to 15 with an average of 6.96 indicating high level of polymorphism. Average major allele frequency for current study was recorded as 0.351, availability 0.615, gene diversity 0.759 and polymorphism percentage 39.84 per cent which resembled the earlier reports of Sheet *et al.,* 2018. The high level of genetic variability using SSR primers demonstrated the suitability of using molecular markers in unraveling the genetic relationships among genotypes of mulberry indigenous to India (Bhattacharya and Ranade, 2001) [3]. Using inter simple sequence repeats (ISSR) markers Kar *et al.,* (2008) [5] analysed the genetic relationships of mulberry accessions in India and found 84 polymorphic bands from 14 primers with the genetic diversity among accessions 0.263 ± 0.094.The occurrence of wide genetic divergence among the mulberry accessions could be attributed to the prevalence of varied agroclimatic conditions in India (Banerjee *et al*., 2007) [2]. In the present study, PIC values ranged from 0.486 (Mul3SSR91) to 0.901 (M6) with an average of 0.724, used to refer the relative value of each marker with respect to the amount of polymorphism exhibited. The PIC values reported in this study were higher than those of Zhao *et al.,* (2009) [8] who studied 27 mulberry accessions using 15 SSR primers. The results of cluster tree grouped 44 genotypes into four main clusters, A, B, C and D with sub-clusters and sub-sub clusters.

Conclusions

The present study helped us to explore the diversity among mulberry genotypes and identifying the highly polymorphic SSR primers. High level of variability between the genotypes could further be exploited through hybridization and selection among identified genotypes for the development of superior mulberry varieties. Genotype NS-1 and NS-2 were identified as duplicates and other genotypes revealed genetic variability to one other level.

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